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OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

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C.-E. A. WINSLOW

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STUDIES RELATIVE TO THE APPARENT CLOSE RELATIONSHIP BETWEEN BACT. PERTUSSIS AND B. BRONCHISEPTICUS[1]

I. CULTURAL AGGLUTINATION AND ABSORPTION REACTIONS

N. S. FERRY AND ARLYLE NOBLE

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Mallory and his associates in 1912 and 1913, while attempting to prove the relationship of *Bact. pertussis* to whooping cough by animal inoculations, found that the problem was much more complicated than anticipated, their interpretations being clouded by the introduction into the question of the *Bacillus bronchisepticus*, as a result of its presence in some of the animals used for experimental purposes.

During the discussion of the paper by Mallory the observation was made by Dr. J. L. Rhea, that the lesions in pertussis in the human being, due to the bacterium of Bordet are similar to the lesions in the dog, which result from an infection with *B. bronchisepticus*, the cause of distemper, and that this fact suggested an interesting relationship between the two organisms. Later, Mallory states,

> Further experimental work is evidently needed in order to clear up the subject. The two organisms closely resemble each other morphologically and in cultures on potato blood agar, but can be distinguished by their difference in motility and their alkali production in litmus milk.

Soon after the appearance of the work of Mallory, the writers started some experimental work with the two organisms in question, to determine, if possible, just how close was this relationship which apparently existed between them.

[1] Presented at Eighteenth Annual Meeting of the Society of American Bacteriologists, New Haven, Conn., December 27–29, 1916.

At first the experiments were undertaken with two strains of *Bact. pertussis* which had been cultured since 1911, one having been furnished by the laboratory of Bordet and the other isolated in our own laboratory, and three strains of *B. bronchisepticus* isolated by one of us (N. S. F.), one from a dog in 1908, one from a monkey in 1912 and one from a human subject in 1913. Later on these strains were augmented by ten strains of *Bact. pertussis*, furnished by Dr. Olga R. Povitzky of the New York Board of Health Laboratory, through the courtesy of Dr. Park, and three strains of *Bact. influenzae*, together with one strain of a *Bact. pertussis*-like organism from pertussis sputum, isolated in our own laboratory.

CULTURAL REACTIONS

When first isolated, the *Bact. pertussis* developes slowly and, as a rule, preferably on special media, as reported by Bordet, Woolstein and others. After several months of repeated transplantings, however, its ability to grow on various media gradually increases until it finally presents a growth almost identical to, and nearly as luxuriant as, that of *B. bronchisepticus*, and by that time can be cultured on ordinary media.

It has been found by the writers that the one great difference between the two organisms lies in their power of locomotion; the *B. bronchisepticus* is motile while the *Bact. pertussis* is nonmotile, several months of attempting to develop a strain that would give some evidence of motility resulting in failure.

While the cultural reactions have been found practically identical, even to the alkali production in litmus milk, contrary to the report of Mallory, and the tan color on potato is shared by both, yet, with the *Bact. pertussis*, these reactions are extremely tardy in making their appearance, usually taking about two or three weeks longer than with the *B. bronchisepticus*. At the end of this time, however, it is practically impossible to differentiate between the cultures of the two organisms.

The following outline will show the characteristics of these organisms, in a general way:

	B. BRONCHISEPTICUS	BACT. PERTUSSIS
Morphology	Very small, slender rod, showing bipolar staining	Small, slender rod, showing marked bipolar staining
Gram	Negative	Negative
Motility	Motile	Non-motile
Agar slant	Translucent, filiform growth	Translucent, filiform growth
Bouillon	*Cloudy.* Older growth with heavy, stringy sediment	*Cloudy.* Older growth with heavy, stringy sediment
Potato	*Tan.* Light tan in twenty-four hours to dark tan in three weeks; medium becoming tanned	*Tan.* Light yellow in twenty-four hours to tan in 3–5 weeks; medium tanned.
Litmus milk	*Alkaline.* Slightly blue at the surface in forty-eight hours. This color proceeds downward, becoming very dark greenish blue in about seven days, while the lower part decolorizes	*Alkaline.* In about 6 days the litmus milk begins to decolorize at the dark bottom of the tube, becomes slightly blue in upper portion in four weeks, and in from eight to ten weeks can scarcely be distinguished from *B. bronchisepticus.*
Litmus-lactose agar	Alkaline (forty-eight hours)	Alkaline (four to six days)
Glucose agar	No gas	No gas
Gelatin	Not liquefied	Not liquefied
Indol	Negative	Negative
Nitrites in nitrate broth	Negative	Negative

AGGLUTINATION REACTIONS

The agglutination reactions of these two organisms have presented some very interesting and rather novel phenomena which, to the writers, suggest at least a distant relationship between them.

In the early part of this year Povitzky and Worth reported the results of some agglutination experiments with these organisms, using a *Bact. pertussis* antiserum only, and concluded that,

B. pertussis strains can be specifically identified from hemoglobinophilic bacilli, pertussis-like bacilli and *B. bronchisepticus*. In no instance was there cross agglutination between these organisms—at least not higher than 1 : 40.

Our work has corroborated that of the authors so far as they have gone. Table 1 gives the results of agglutination between anti-

TABLE 1

Agglutination tests between antipertussis serum and heterologous suspensions. Results August 31, 1916. Serum from rabbit 7, treated with Bact. pertussis no. 0363 (Bordet)

DILUTIONS	SUSPENSIONS OF								
	Bact. pertussis		B. bronchisepticus			Pertussislike bacteria	Bact. influenzae		
	No. 0363 (Bordet)	No. 0590 (Ferry)	No. 36 (Dog)	No. 123 (Monkey)	(Human)	No. 032558	T. b. No. 2	"S"	No. 3
1–10	–	–	–	–	–	+	–	–	+
1–20	–	–	–	–	–	–	–	–	–
1–40	–	+	–	–	–	–	–	–	–
1–80	+	++	–	–	–	–	–	–	–
1–200	+++	+++	–	–	–	–	–	–	–
1–400	+++	+++	–	–	–	–	–	–	–
1–800	+++	+++	–	–	–	–	–	–	–
1–1600	+++	+++	–	–	–	–	–	–	–
1–2000	+++	+++							
1–3200	+++	+++							
1–6400	++	+++							
1–10000	+	++							
1–20000	–	+							
1–40000	–	–							
Control	–	–	–	–	–	–	–	–	–

This table and the following one illustrate the presence of pro- agglutinoids in *Bact. pertussis* and *B. bronchisepticus* antiserum making it necessary to test all normal and immune sera in dilutions higher than 1–80.

pertussis serum and suspensions of *Bact. pertussis*, a pertussis-like bacillus, *B. bronchisepticus*, and three hemoglobinophilic bacilli. But on using a *B. bronchisepticus* antiserum, the results are entirely different, as the *Bact. pertussis* suspensions agglutinate

nearly as well as the homologous suspension, the *B. bronchisepticus*. The results of such a test are given in table 2 where agglutination tests were made with suspensions of fourteen strains of *Bact. pertussis* and an homologous suspension of *B. bronchisepticus* against antibronchisepticus serum. And finally, table 3 is a summary of all the homologous and cross agglutination tests.

The results therefor show that the *B. bronchisepticus* antiserum will agglutinate both the *B. bronchisepticus* and *Bact. pertussis* antigens, while the *Bact. pertussis* antiserum will agglutinate only its homologous antigen, the *Bact. pertussis*. This reaction was characteristic of every strain of *Bact. pertussis* and *B. bronchisepticus* under observation.

Whether this shows a true relationship between the two organisms, and can be called a specific reaction, is a question.

Preparation of the antigens for the production of the antisera. *B. bronchisepticus* was grown on plain agar, *Bact. pertussis* no. 0363 (Bordet) and 0590 (Ferry) on ascitic agar, and the New York strains of *Bact. pertussis* and the hemoglobinophilic bacilli on whole-blood (rabbit) agar. Twenty-four hour growths were washed off in 0.2 per cent trikresol in physiologic salt solution—1 cc. to a culture of *Bact. influenzae*, 2.5 cc. to a culture of *Bact. pertussis* and 5 cc. to a culture of *B. bronchisepticus*. The suspensions were thoroughly shaken in a mechanical shaker and, after two days, tested for sterility.

Production of antisera. Before being treated, the serum of each rabbit was tested for agglutinins against all of the organisms under discussion. Any animal showing an agglutination higher than 1–20 against *B. bronchisepticus* antigen or 1–40 against any of the other organisms, was not used.

The rabbits were given three intravenous injections of increasing doses from 0.5 cc. to 2 cc. three days apart and were bled on the fourth day after the last dose; 0.2 per cent trikresol was added to the serum to insure sterility.

Preparation of suspensions for agglutination tests. The suspensions were prepared in general, as follows:

Each culture was transplanted daily for from three days to three weeks—depending upon the organism—on media best suited to it, to insure a good vigorous growth. Then twenty-four hour cultures of

TABLE 2

Agglutination tests showing presence of pertussis agglutinins in antibronchisepticus serum. Results November 7, 1916. Serum from rabbit 1, treated with B. bronchisepticus no. 36 (dog)

DILUTIONS	SUSPENSION OF														
	B. bronchisepticus	Bact. pertussis													
	No. 36 (Dog)	No. 0363 (Bordet)	No. 55	No. 93	No. 95	No. 98	No. 100	No. 109	No. 110	No. 114	No. 154	No. 163	No. 248	No. 251	No. 253
1-10	++	++	++	−	+	+	+	+	++	+	+	−	+	−	+
1-20	+++	++	+++	+	++	++	+	++	++	+	+	−	+	−	++
1-40	+++	+++	+++	++	+++	+++	++	++	+++	+	++	+	++	+	++
1-80	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++
1-200	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1-400	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1-800	+++	+++	+++	+++	++	+++	++	+++	++	+++	+++	+++	++	++	+++
1-1600	+++	+	++	+	+	++	+	++	+	+++	++	+	+	+	++
1-2000	+++	−	−	+	−	+	+	+	−	+++	+	+	−	−	+
1-3200	+++	−	−	−	−	−	−	−	−	+	−	−	−	−	−
1-6400	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1-10000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1-20000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1-40000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Agglutination tests of B. bronchisepticus, Bact. pertussis, and Bact. influenzæ suspensions against homologous and heterologous sera

ANTI-SERA	RABBIT NO.	SUSPENSIONS																						
		B. bronchisepticus			Bact. pertussis																Pertussis-like bac.	Bact. influenzæ		
		No. 36 (Dog)	No. 123 (Monkey)	(Human)	No. 0363 (Bordet)	No. 0590 (Ferry)	No. 55	No. 93	No. 95	No. 98	No. 100	No. 109	No. 110	No. 114	No. 154	No. 163	No. 248	No. 251	No. 253	No. 032558	T.b.2	"S"	No. 3
B. bronchisepticus																								
No. 36.....	1	**1-6400**	1-6400	1-6400	1-1600	1-800	1-1600	1-2000	1-1600	1-2000	1-2000	1-2000	1-1600	1-3200	1-2000	1-2000	1-1600	1-1600	1-1600	1-10	1-40	–	–
	2	**1-10000**	1-20000	1-10000	1-400	1-400	1-2000	1-800												1-20	1-10	–	1-20
No. 123....	3	1-6400	**1-10000**	1-20000	1-3200	1-3200	1-20000	1-6400												1-20	1-10	–	–
	4	1-10000	**1-10000**	1-20000	1-3200	1-6400	1-6400	1-3200	1-2000	1-6400	1-3200	1-6400	1-3200	1-3200	1-6400	1-6400	1-3200	1-3200	1-6400	1-20	–	–	1-80
(Human)..	5	1-2000	1-3200	**1-6400**	1-800	1-2000	1-800	1-800	1-800	1-2000	1-1600	1-2000	1-2000	1-1600	1-2000	1-2000	1-800	1-800	1-2000	–	–	–	1-10
	6	1-10000	1-10000	**1-10000**	1-800	1-800	1-800	1-800												1-10	–	–	1-10
Bact. pertussis:																								
No. 0363...	7	–	–	–	**1-10000**	1-20000	1-10000	1-20000	1-800	1-10000	1-10000	1-20000	1-10000	1-20000	1-20000	1-20000	1-10000	1-10000	1-20000	1-10	–	–	1-10
	8	1-80	1-80	1-80	**1-10000**	1-20000	1-40000	1-20000												–	–	–	1-20
No. 0590...	9	–	1-10	–	1-6400	**1-6400**	1-20000	1-10000												1-20	1-10	–	–
	10	1-10	1-10	–	1-6400	**1-10000**	1-6400	1-10000												1-20	–	–	1-10
No. 55.....	19	–	–	–		1-40000	**1-20000**																
No. 93.....	20	–	–	–		1-20000		**1-20000**															
No. 95.....	21	–	1-10	1-10		1-20000			**1-3200**														
No. 98.....	22	–	–	–	1-20000					**1-20000**													
No. 100....	23	1-20	1-40	1-20	1-20000						**1-20000**												
No. 109....	24	–	–	–	1-10000							**1-10000**											
No. 110....	25	1-10	1-10	1-10		1-10000							**1-20000**										
No. 114....	26	1-10	–	–		1-20000								**1-20000**									
No. 154....	27	1-10	1-10	–		1-20000									**1-20000**								
No. 163....	28	1-10	1-10	1-20		1-20000										**1-20000**							
No. 248....	29	1-20	1-20	1-20		1-10000											**1-20000**						
No. 251....	30	1-40	1-80	1-40	1-2000													**1-3200**					
No. 253....	31	1-20	1-10	1-10	1-6400														**1-20000**				
Bact. pertussis-like:																							
No. 032558.	11	1-10			–	–														**1-20000**	–	–	1-10
Bact influenzæ:																							
T.b.2.....	13	–	1-20	–	–	–	–	1-10												1-10	**1-3200**	1-20	1-80
"S".....	15	1-20	1-20	1-10	1-10	1-20		1-20												1-40	1-200	**1-6400**	1-200
No. 3.....	17	1-20	1-20	1-20	1-20	1-20		1-40												1-20	1-10	1-20	**1-1600**

TABLE 2

Agglutination tests showing presence of pertussis agglutinins in antibronchisepticus serum. Results November 7, 1916. Serum from rabbit 1, treated with B. bronchisepticus no. 36 (dog)

DILUTIONS	SUSPENSION OF														
	B. bronchisepticus	Bact. pertussis													
	No. 36 (Dog)	No. 0363 (Bordet)	No. 55	No. 93	No. 95	No. 98	No. 100	No. 109	No. 110	No. 114	No. 154	No. 163	No. 248	No. 251	No. 253
1-10	++	++	++	−	+	+	+	+	++	+	+	−	+	−	+
1-20	+++	++	+++	+	++	++	+	++	++	+	+	−	+	−	++
1-40	+++	+++	+++	++	+++	+++	++	++	+++	+	++	+	++	+	++
1-80	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++
1-200	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1-400	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1-800	+++	+++	+++	+++	++	+++	++	+++	++	+++	+++	+++	++	++	+++
1-1600	+++	+	++	+	+	++	+	++	+	+++	++	+	+	+	++
1-2000	+++	−	−	+	−	+	+	+	−	+++	+	+	−	−	+
1-3200	+++	−	−	−	−	−	−	−	−	+	−	−	−	−	−
1-6400	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1-10000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1-20000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1-40000	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 3

Agglutination tests of B. bronchisepticus, Bact. pertussis, and Bact. influenzae suspensions against homologous and heterologous sera

		SUSPENSIONS		
		B. bronchisepticus	Bact. pertussis	Bact. influenzae
Rabbit 1	Bronchisepticus serum	++++	+	−
	Pertussis serum	+	++++	−
	Influenzae serum	−	−	++++
Rabbit 2	Bronchisepticus serum	++++	+	−
	Pertussis serum	+	++++	−
	Influenzae serum	−	−	++++
Rabbit 3	Bronchisepticus serum	++++	+	−
	Pertussis serum	+	++++	−
	Influenzae serum	−	−	++++

TABLE 3

Agglutination tests of B. bronchisepticus, Bact. pertussis, and Bact. influenzae suspensions against homologous and heterologous sera

ANTI-SERA	RABBIT NO.	SUSPENSIONS																					
		B. bronchisepticus			Bact. pertussis															Pertussis-like bac.	Bact. influenzae		
		No. 36 (Dog)	No. 123 (Monkey)	(Human)	No. 0363 (Bordet)	No. 0590 (Ferry)	No. 55	No. 93	No. 95	No. 98	No. 100	No. 109	No. 110	No. 114	No. 154	No. 163	No. 246	No. 251	No. 253	No. 032558	T.b.2	"S"	No. 3
B. bronchisepticus																							
No. 36	1	1-6400	1-6400	1-6400	1-1600	1-800	1-1600	1-2000	1-1600	1-2000	1-2000	1-2000	1-1600	1-3200	1-2000	1-2000	1-1600	1-1600	1-1600	1-10	1-40	–	–
	2	1-10000	1-20000	1-10000	1-400	1-400	1-2000	1-800												1-20	1-10	–	1-20
No. 123	3	1-6400	1-10000	1-20000	1-3200	1-3200	1-20000	1-6400												1-20	1-10	–	–
	4	1-10000	1-10000	1-20000	1-3200	1-6400	1-6400	1-3200	1-2000	1-6400	1-3200	1-6400	1-3200	1-3200	1-6400	1-6400	1-3200	1-3200	1-6400	1-20	–	–	1-80
(Human)	5	1-2000	1-3200	1-6400	1-800	1-2000	1-800	1-800	1-800	1-2000	1-1600	1-2000	1-2000	1-1600	1-2000	1-2000	1-800	1-800	1-2000	–	–	–	1-10
	6	1-10000	1-10000	1-10000	1-800	1-800	1-800	1-800												1-10	–	–	1-10
Bact. pertussis:																							
No. 0363	7	–	–	–	1-10000	1-20000	1-10000	1-20000	1-800	1-10000	1-10000	1-20000	1-10000	1-20000	1-20000	1-20000	1-10000	1-10000	1-20000	1-10	–	–	1-10
	8	1-80	1-80	1-80	1-10000	1-20000	1-40000	1-20000												–	–	–	1-20
No. 0590	9	–	1-10	–	1-6400	1-6400	1-20000	1-10000												1-20	1-10	–	–
	10	1-10	1-10	–	1-6400	1-10000	1-6400	1-10000												1-20	–	–	1-10
No. 55	19	–	–	–		1-40000	1-20000																
No. 93	20	–	–	–		1-20000		1-20000															
No. 95	21	–	1-10	1-10		1-20000			1-3200														
No. 98	22	–	–	–	1-20000					1-20000													
No. 100	23	1-20	1-40	1-20	1-20000						1-20000												
No. 109	24	–	–	–	1-10000							1-10000											
No. 110	25	1-10	1-10	1-10		1-10000							1-20000										
No. 114	26	1-10	–	–		1-20000								1-20000									
No. 154	27	1-10	1-10	–		1-20000									1-20000								
No. 163	28	1-10	1-10	1-20		1-20000										1-20000							
No. 246	29	1-20	1-20	1-20		1-10000											1-20000						
No. 251	30	1-40	1-80	1-40	1-2000													1-3200					
No. 253	31	1-20	1-10	1-10	1-6400														1-20000				
Bact. pertussis-like:																							
No. 032558	11	1-10			–	–														1-20000	–	–	1-10
Bact influenzae:																							
T.b.2	13	–	1-20	–	–	–	–	1-10												1-10	1-3200	1-20	1-80
"S"	15	1-20	1-20	1-10	1-10	1-20		1-20												1-40	1-200	1-6400	1-200
No. 3	17	1-20	1-20	1-20	1-20	1-20		1-40												1-20	1-10	1-20	1-1600

these were planted on plain agar in quart whiskey flasks—except in the case of *Bact. influenzae* for which whole-blood agar was used. It has been found advantageous to use agar in whiskey flasks, as this method not only gives a larger amount of suspension for less labor, but it also gives a far heavier and healthier growth than when tubes are used, probably because of the greater supply of media, moisture and air. An abundant growth of *Bact. pertussis* can be obtained on plain agar in flasks when it will grow only slightly or not at all on plain agar in tubes. And a young, vigorous growth is necessary to the production of homogeneous suspensions. Also in using agar without either ascitic fluid or blood, all possibility of clumping from this source is avoided. In the case of the influenza bacillus it is necessary to use blood agar to obtain any growth. Just enough whole blood is added to agar to insure growth and it is used before any hemolysis takes place, in order that the suspension may contain as little blood as possible.

The flasks were incubated for from eighteen to twenty-four hours and the growth washed off with 0.5 per cent formalin in physiologic salt solution. The suspensions were then shaken for a few hours and later, after being tested for sterility, were filtered through paper and standardized to about 2000 million per cubic centimeter.

With this technique, homogeneous suspensions of all the organisms used, were produced.

Agglutination tests. In carrying out the tests, the serum was diluted with physiologic solution and each tube contained 0.5 cc. suspension plus 0.5 cc. diluted serum. The tests were all macroscopic and were incubated at 37°C. for twenty-four hours. (+++) represents complete agglutination with fluid clear; (++) partial agglutination with marked clumping, but fluid not entirely cleared up; (+) slight agglutination, but still with positive clumping; and (−) no clumping, no clearing.

Specially graduated 1 cc. pipettes were used for making the serum dilutions and a different pipette was used for each dilution. All glassware used in connection with the tests was clean and sterile.

AGGLUTINATION REACTIONS WITH SERUM FROM DISTEMPER RABBITS AND DOGS

In testing apparently normal rabbits for agglutinins, before beginning inoculation for the production of antisera, we found that if a serum agglutinated *B. bronchisepticus* in a dilution higher

than 1–20, it also agglutinated *Bact. pertussis* and generally in higher dilutions. In one instance when there was no agglutination against *B. bronchisepticus*, the serum agglutinated *Bact. pertussis* in a dilution of 1–400 (rabbit E) (see table 4).

A few of these rabbits subsequently developed symptoms of distemper; the others may have recovered from an attack.

Sera from only two distemper dogs have been tested, but, with these, similar results were obtained. As in rabbits, agglutinins for *Bact. pertussis* were manifest in higher dilutions than for *B. bronchisepticus*. These dogs exhibited typical symptoms of distemper and were in the later stages of the disease.

Dog 1 agglutinated *B. bronchisepticus* no. 36 (dog) at 1–20; and *Bact. pertussis* no. 0363 (Bordet), 1–400 and no. 93, 1–1000. Dog 2 agglutinated *B. bronchisepticus* no. 36 (dog) at 1–80; and *Bact. pertussis* no. 0363, 1–1000 and no. 93, 1–1000.

On absorption with *B. bronchisepticus* the agglutinins for that organism are removed, while the agglutinins for *Bact. pertussis* are affected little or not at all. On absorption with *Bact. pertussis*, the pertussis agglutinins are removed, while those for *B. bronchisepticus* are unaffected.

ABSORPTION REACTIONS

Upon submitting to absorption tests those antiserums which were produced by the injection of *B. bronchisepticus* antigen into rabbits, and which were found to agglutinate both the *B. bronchisepticus* and *Bact. pertussis* suspensions, the following results were obtained.

Upon absorbing with *B. bronchisepticus* suspension, the agglutinins for *B. bronchisepticus* (the major agglutinins) were absorbed, but the *Bact. pertussis* agglutinins (the minor agglutinins) were still intact (table 5), and an absorption with *Bact. pertussis* was necessary before they were neutralized. In other words, the *B. bronchisepticus* antigen stimulated the formation of both *B. bronchisepticus* and *Bact. pertussis* agglutinins, but contrary to what one would expect, was not able to absorb the *Bact. pertussis* agglutinins, (the minor agglutinins). It was also found, in the

TABLE 4

Agglutinin reaction in rabbits not used for production of anti-sera

DILUTIONS	RESULTS																					
	Rabbit A		Rabbit B		Rabbit C		Rabbit D		Rabbit E		Rabbit F		Rabbit G		Rabbit H		Rabbit I					
	B. bronchiseptic. tieus no. 36	B. pertussis no. 55	B. bronchiseptic. tieus no. 36	B. pertussis no. 55	B. bronchiseptic. tieus no. 36	B. pertussis no. 109	B. bronchiseptic. tieus no. 36	B. pertussis no. 114	B. bronchiseptic. tieus no. 36	B. pertussis no. 163	B. bronchiseptic. tieus no. 36	B. pertussis no. 163	B. bronchiseptic. tieus no. 36	B. pertussis no. 248	B. bronchiseptic. tieus no. 36	B. pertussis no. 251	B. bronchiseptic. tieus no. 36	B. pertussis no. 253				
1–10	+	−	+	+	+	+	+	+	−	−	+	−	+	+	+	−	+	+				
1–20	+	+	+	+	+	+	+	+	−	−	+	−	+	+	+	−	+	+				
1–40	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+				
1–80	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+				
1–200	+	+	+	+	−	+	+	+	−	+	+	+	+	+	−	−	+	+				
1–400	−	+	+	+	−	+	+	+	−	+	−	−	+	+	−	−	+	+				
1–800	−	+	−	+	−	+	+	+	−	+	−	−	−	+	−	−	+	+				
1–1600	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+				
Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−				

case of dogs suffering with distemper, that the serum agglutinated *Bact. pertussis* antigen in higher dilutions than the *B. bronchisepticus* antigen. Absorption with *B. bronchisepticus* antigen took out only the *B. bronchisepticus* agglutinin, while it was necessary to absorb with *Bact. pertussis* antigen before the *Bact. pertussis* agglutinin was neutralized (see table 7). The *Bact. pertussis* agglutinins, therefore, were fixed or stable, as far as the *B. bronchisepticus* was concerned, but absorbable for the *Bact. pertussis*, and this type of an agglutinin, which can be produced

TABLE 5

Serum from rabbit 1, treated with B. bronchisepticus absorbed (1-40) with B. bronchisepticus

DILUTIONS	AGGLUTINATION			
	Before absorption		After absorption	
	B. bronchisepticus No. 36	Bact. pertussis No. 0590	B. bronchisepticus No. 36	Bact. pertussis No. 0590
1-80	+++	+++	++	+++
1-200	+++	+++	−	+++
1-400	+++	+++	−	+++
1-800	+++	++	−	++
1-1600	+++	−	−	−
1-2000	+++	−	−	−
1-3200	++	−	−	−
1-6400	+	−	−	−
1-10000	−	−	−	−
Control	−	−	−	−

by one antigen to be taken up or absorbed more readily by another, has been called by the writers, for the lack of a better term, a transitive agglutinin.

The antisera were identical to those used for the agglutination tests.

The heavy suspensions used for absorption were made from twenty hour growths on plain agar in whiskey flasks, suspended in .85 per cent salt solution to which had been added 0.5 per cent formalin. About 10 cc. salt solution was used to a flask for *B. bronchisepticus* and 4 cc. for *Bact. pertussis*. The suspensions were shaken over night in a mechanical shaker and then strained through mull. Each strain had

been transplanted daily for several days before using so that very heavy growths were obtained.

Absorption tests. Equal parts of heavy suspension and serum were mixed; incubated at 37°C.; then centrifugalized and the supernatant

TABLE 6

Absorption tests with B. bronchisepticus and Bact. pertussis antisera

SERUM	ABSORBED WITH	AGGLUTINATION AFTER ABSORPTION	
		B. bronchi-septicus	Bact. pertussis
Antibronchisepticus.			
No. 36 (dog), rabbit 1	*(Original titre)*	*1–6400*	*1–800*
	B. bronchisepticus no. 36	—	—
	Bact. pertussis no. 0590	1–6400	1–80
No. 36 (dog), rabbit 2	*(Original titre)*	*1–10000*	*1–400*
	B. bronchisepticus no. 36	—	—
	Bact. pertussis no. 0590	1–10000	—
No. 123 (monkey), rabbit 3	*(Original titre)*	*1–10000*	*1–3200*
	B. bronchisepticus no. 123	—	1–2000
	Bact. pertussis no. 0590	1–10000	1–80
No. 123 (monkey), rabbit 4	*(Original titre)*	*1–20000*	*1–3200*
	B. bronchisepticus no. 123	—	1–80
	Bact. pertussis no. 0590	1–6400	—
Human, rabbit 5	*(Original titre)*	*1–6400*	*1–800*
	B. bronchisepticus (Human)	—	1–800
	Bact. pertussis no. 0363	1–6400	—
Human, rabbit 6	*(Original titre)*	*1–10000*	*1–800*
	B. bronchisepticus (human)	—	1–800
	Bact. pertussus no. 0363	1–10000	1–80
Antipertussis			
No. 0590, rabbit 10	*(Original titre)*	*1–10*	*1–10000*
	Bact. pertussis No. 0590		—
	B. bronchisepticus No. 36		1–6400

fluid tested for agglutinins. The time of incubation, the number of absorptions and the dilution of the serum were varied in a number of ways. For example, we found that when either antipertussis or antibronchisepticus serum was diluted to 1–40, we obtained a more nearly

complete absorption than when diluted 1–10, which may be due to the fact that complete agglutination does not occur in the lower dilutions with either of the organisms, especially with *Bact. pertussis*. And in the absorption tests, it was noted that clumping and clearing was not so complete at 1–10 or 1–20 as at 1–40.

Again, serum from rabbit 5, treated with *B. bronchisepticus* (Human) was absorbed as many as four times, the dilutions being from 1–5 to 1–40 and the tests being shaken in a mechanical shaker before each incubation, with no effect on the agglutinins for *Bact. pertussis*.

It was found that when antibronchisepticus serum was absorbed with *B. bronchisepticus* sufficiently to remove the agglutinins for *B. bronchi-*

TABLE 7

Absorption tests with serum from distemper dogs

SERUM	ABSORBED WITH	AGGLUTINATION AFTER ABSORPTION		
		B. bronchisepticus no. 36 (dog)	Bact. pertussis	
			No. 0363	No. 93
Dog 1	*(Original titre)*	*1–20*	*1–400*	*1–1000*
	B. bronchisepticus no. 36	—	1–200	1–1000
	Bact. pertussis no. 0363	1–20	—	1–20
Dog 2	*(Original titre)*	*1–80*	*1–1000*	*1–1000*
	B. bronchisepticus no. 36	—	1–800	
	Bact. pertussis no. 0363	1–80	—	

septicus the agglutinins for *Bact. pertussis* were still unaffected. This could be done by an absorption at 1–40, incubated 24 hours. Table 5 gives the results of such an experiment.

But it required repeated absorption with *B. bronchisepticus* before any marked effect was produced in the pertussis agglutinins, and this happened only with the dog strain.

Finally, the following method was used with the six antibronchisepticus sera. The results of these experiments are given in table 6.

Each serum was absorbed three times with its homologous antigen—that is, serum from rabbit treated with *B. bronchisepticus* (dog) was absorbed with the dog strain—and with *pertussis* antigen as follows:

First absorption, serum 1–10 incubated two hours.
Second absorption, serum 1–20 incubated two hours.
Third absorption, serum 1–40 incubated eighteen hours.

SUMMARY AND DISCUSSION

1. After repeated transplantings *Bact. pertussis* has been found to give the same cultural reactions as *B. bronchisepticus*—the tan growth on potato and the alkaline reaction in litmus mik being the most prominent characteristics. The difference in motility and the tardiness in the reactions of *Bact. pertussis* on culture media are differential characteristics.

2. *B. bronchisepticus* antiserum agglutinates not only *B. bronchisepticus* (1–6400 to 1–2,000), but also *Bact. pertussis* (1–400 to 1–6400).

3. *Bact. pertussis* antiserum on the other hand agglutinates only the pertussis bacillus.

4. There was no cross agglutination between *B. bronchisepticus* and a pertussis-like bacillus or three hemoglobinophilic bacilli. Also, there was no agglutination between *Bact. pertussis* and these organisms.

5. *Bact. pertussis* antiserum of high agglutination titre (1–3200 to 1–20000) has been produced, in rabbits, by three intravenous inoculations (three days apart) of sterile, unheated vaccines of fifteen strains of *Bact. pertussis.*

Povitzky and Worth, in the article cited above, conclude that,

> A strongly agglutinating pertussis serum was best obtained in the rabbit by ten to twelve intraperitoneal inoculations of living cultures at seven-day intervals. Agglutinins are also produced by vaccines, but not as abundantly as by living cultures.

After ten to twelve inoculations of live cultures, their rabbits show a titre of from 2400 to 5000 and one rabbit went to 10000.

Over 50 per cent (9 out of 17) of our rabbits show a titre of 20000, and only two were as low as 3200. The one rabbit in the tables of Povitsky and Worth treated with killed vaccine (heated) shows a titre of 1600 after the sixth inoculation and no agglutination after the fourth. They also say,

Two rabbits, more responsive to vaccines, developed, after a few inoculations, a comparatively high agglutination titre—up to 1000.

6. Sera from rabbits treated wtih *Bact. pertussis* grown on blood media developed marked agglutinins for *Bact. pertussis* grown on plain agar.

This is contrary to Bordet's statement that animals inoculated with *Bact. pertussis* grown on Bordet-Gengou medium develop agglutinins for *Bact. pertussis* grown on the same media, not for the organism grown on plain agar; and is in accordance with Povitzky's and Worth's conclusion that,

> From our experience it would seem that the culture medium influences an agglutinable strain in so far as it affects its growth and best development, not in its production of different kinds of agglutinins.

Also, serum from a rabbit treated with *Bact. pertussis* no 0363 (Bordet) grown on *ascitic agar* for the past three years agglutinates all thirteen strains grown on *blood media* (with only one generation, for suspensions, on plain agar); and sera from rabbits treated with thirteen strains grown on blood media agglutinate the ascitic agar strains.

7. When *B. bronchisepticus* antiserum is absorbed with *B. bronchisepticus* sufficiently to remove the agglutinins for *B. bronchisepticus*, the agglutinins for *Bact. pertussis* are still unaffected. These unaffected or fixed agglutinins have been called by the writers, transitive agglutinins.

Upon repeated absorption, agglutinins for *Bact. pertussis* have been removed by the dog strain of *B. bronchisepticus*, slightly reduced by the monkey strain and affected not at all by the human strain.

It may be that, by the absorption tests, grades of differences are brought out between *B. bronchisepticus* from dog, monkey and human which are not shown in the agglutination tests. A similar differentiation is thought to have been brought out by complement fixation tests. (See article in press by Ferry and Klix).

8. When *B. bronchisepticus* antiserum is absorbed with *Bact. pertussis*, agglutinins for *Bact. pertussis* are removed, but agglutinins for *B. bronchisepticus* are unaffected.

9. When *Bact. pertussis* antiserum is absorbed with *Bact. pertussis*, agglutinins for that organism are removed.

10. When *Bact. pertussis* antiserum is absorbed with *B. bronchisepticus*, agglutinins for *Bact. pertussis* are unaffected.

11. The similar morphology, the identical cultural reactions on differential media, the presence of *Bact. pertussis* agglutinins in artificially produced antibronchisepticus serum and in serum from dogs and rabbits suffering or recovered from distemper, all point toward a close relationship between *Bact. pertussis*, the cause of whooping cough and *B. bronchisepticus*, the cause of distemper.

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THE GROWTH OF BACTERIA IN PROTEIN-FREE ENZYME- AND ACID-DIGESTION PRODUCTS

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The advantages of protein-free synthetic media for biochemical study were appreciated early in the history of bacteriology. With a medium the chemical composition of which is known it should be possible to determine by exact study the chemical nature of the products of bacterial growth. In the complex peptone and infusion media this is impossible.

Many synthetic media have been devised, most of them containing several inorganic salts, and having glucose or glycerol as the source of carbon, and organic ammonium salts, asparagin or glycocoll as the source of nitrogen. Among the most important of the earlier media were those of Cohn, Nägeli, Fränkel, and Uschinsky. The chief difficulty with all of these, however, is that only a limited number of bacteria will grow in them. The more delicate and fastidious of the pathogenic class apparently do not find proper nutriment in such simple media. *B. diphtheriae* either refuses to develop or grows very slowly. Many other organisms show the same indifference.

With the discovery of the value of protein digestion products, and particularly the amino acids, in animal nutrition, these substances assumed much importance in the field of bacterial metabolism. It has been demonstrated that all proteins must first be reduced by the digestive secretions to the amino acid stage before they can be utilized in animal cell metabolism. Based on a similar assumption, protein hydrolysis products and mixtures of amino acids have been in a few instances used as culture media for bacteria.[1]

[1] For a somewhat comprehensive historical review of this subject the reader is referred to the doctorate thesis of one of the authors (Robinson) in the Yale University Library.

Apparently the first protein-free medium which compared favorably with the universally-used peptone medium was the opsine studied by Dalimier and Lancereaux (1913). Their paper is very suggestive, and in a large measure served as the inspiration and point of departure of the present investigation.

Opsine is a biuret-free product resulting from the combined action of trypsin, erepsin and pepsin on certain protein materials, not named by the authors or manufacturers.[2] It contains the amino acids of the hydrolyzed proteins, also nucleic acid and glucosamine, or their decomposition products. Phosphorus would presumably be present in the nuclein and sulphur in the cystine. The opsine gave a high formol titration for monamino acids. Leucine and tyrosine were detected by their crystals, and tryptophane by the bromine water test. The biuret test for protein was always negative. On this basic medium, with the addition of glycerol and agar, Dalimier and Lancereaux, successfully cultivated not only the more common pathogens and non-pathogens, but also such delicate organisms as the pneumococcus, gonococcus, meningococcus, *B. diphtheriae*, *B. tetani* and *B. tuberculosis* (human, bovine and avian types). These were all carried through several transplants, without any apparent loss of viability. When injected into animals, sterile opsine had no injurious effect.

EXPERIMENTAL

In the present investigation numerous cultural tests were made with opsine as the chief and, as a rule, the only source of nitrogen in the medium. Cultural studies were also carried on with protein-free acid-digestion products of proteins, as will be shown later in the paper.

Opsine as a culture medium

The opsine was obtained from Paris in small metal capsules. In its original state it resembles highly concentrated commercial meat extract, although much lighter in color. It dissolves readily

[2] M. Grémy, Rue de la Tour d'auvergne, Paris.

in water, yielding a brown but perfectly clear solution which is easily and completely decolorized by filtration through animal charcoal. The following tests were made with the decolorized solution.

Different modifications of the biuret method for the presence of proteins gave negative results, thus bearing out the contention of Dalimier and Lancereaux that the product is protein-free. The bromine water test for tryptophane was always negative. This observation is opposed to that of the previous authors, who claimed that tryptophane is present. Millon's Reagent gave a very weak reaction. No tyrosin crystals could be identified upon slow evaporation of a strong solution of the opsine, but leucine crystals were very numerous.

In the Sörensen formol titration, 5 cc. of 1 per cent opsine solution required 3.5 cc. of N/20 NaOH to neutralize it after the addition of neutral formalin. In other words, 1 gram of opsine contained 49 mgm. of nitrogen as monamino acid. A 1 per cent solution of opsine has a reaction with phenolphthalein of about + 1.

The first experiments were conducted with the opsine media as prepared by Dalimier and Lancereaux. Since opsine is so readily soluble, the different media were prepared with ease. For opsine bouillon, opsine and sodium chloride were added to the water and the mixture heated for a few minutes, or until solution was complete. The reaction was then adjusted, and the medium boiled over a flame for a few minutes and filtered. In the preparation of agar, bouillon was first made as described, and the desired amount of agar added. The agar medium was always tubed without filtering. Witte's powdered agar was employed. All of the media were sterilized for fifteen minutes at 12 pounds of extra steam pressure.

Inoculations were made from twenty-four hour slant agar cultures, and the growths recorded as follows:

−	No growth
±	Very scanty
+	Scanty
+±	Sparse
++	Moderate
++±	Abundant
+++	Luxuriant growth

This system was employed in all of the experiments. In estimating the growth of any particular organism a mental comparison was made with the average growth of that organism on the corresponding extract-peptone medium. This standard growth was rated as +++. These comparisons are, of course, only roughly approximate. Special care was taken, however, to make the inoculations light and as nearly alike as possible. In duplicate experiments the growths agreed surprisingly well. Only a few of the tables are presented in this paper. For a complete record the reader is referred to the original thesis in the Yale Library.

In the following experiments the media had the same composition as those employed by Dalimier and Lancereaux.

Medium I		*Medium II*	
Opsine 1.0 per cent NaCl 0.5 per cent	faintly acid to litmus	Opsine 1.0 per cent NaCl 0.5 per cent Glycerol 5.0 per cent	faintly acid to litmus
Medium III		*Medium IV*	
Opsine 1.0 per cent NaCl 0.5 per cent	faintly alkaline to litmus	Opsine 1.0 per cent NaCl 0.5 per cent Glycerol 5.0 per cent	faintly alkaline to litmus

Each of the media was employed with and without agar, the amount of agar in the solid media being 1.5 per cent. As test organisms 12 of the common and representative pathogens, and 5 non-pathogens were used. The following is a summary of the results.

The alkaline was the more favorable of the two reactions for *M. cholerae, B. anthracis, B. abortus, Str. pyogenes* and the diphtheria group, and the acid for *B. pyocyaneus, Staph. aureus* and *B. typhi.* On the opsine agar the growths were much better than in the opsine bouillon, being in general almost equal to those on the common meat extract peptone agar, and slightly better than those on a medium of Witte's peptone 1 per cent., NaCl 0.5 per cent, agar 1.5 per cent. Glycerol in the alkaline

bouillon slightly increased the growth of almost all of the organisms, but in the acid bouillon it was more often detrimental. This can probably be attributed to the action of acids produced from it by the organisms. In agar the glycerol nearly always appeared to increase the growth slightly. It was also very favorable to intense pigment production.

The addition of Liebig's meat extract, 0.5 per cent, to neutral opsine agar, yielded considerably better results than the opsine neutral agar alone. The difference between the two was more pronounced at forty-eight hours than at twenty-four. The growths on this extract medium were as a rule almost, or quite, as good as on the common laboratory media. Of course, such a medium would not be protein-free, as Liebig's meat extract has been shown to contain traces of proteoses and peptones.

Some of the more fastidious organisms grew on a faintly alkaline medium of fresh beef infusion agar + 1 per cent opsine almost as well as on meat infusion peptone agar. The following table shows the growths of the third transplants on this medium after thirty hours' incubation:

Gonococcus (287)	+*
Pneumococcus (M)	++ large discrete colonies
Pneumococcus (697)	+* small crowded colonies
Meningococcus (M)	++
Meningococcus (200)	++*
B. pertussis	++*
B. diphtheriae (Y. M. S.)	+++
Str. pyogenes	++*
M. melitensis	+

When glucose (1 per cent) was added to opsine agar, neutral to litmus, the growths were usually less abundant than on the plain opsine agar. In the case of *Streptococcus pyogenes*, *B. pyocyaneus*, and *B. prodigiosus*, however, the reverse was true. Like glycerol, glucose greatly increased and intensified color production by the last two organisms. The inhibition on the part of the glucose was presumably due to acid formation.

Nitrates were reduced to nitrites in decolorized opsine bouillon by the five strains of *B. coli* and the one of *B. aerogenes* tried. The sterile controls were negative. As might be expected

from the negative tryptophane tests, no indol could be detected in coli cultures even in 2 per cent decolorized opsine. There was always good growth, and the peptone water controls of the same organisms were strongly indol-positive. Tests were made after three and five days of incubation.

Unfiltered cultures of *B. coli* in 1 per cent opsine gave the biuret and the Hopkins and Cole protein tests after only two days of incubation. It is interesting to note that in some old *B. coli* cultures which had been kept in the laboratory for about two months, the clear supernatant fluid gave a negative biuret test, while the sediment was positive. This would seem to indicate that in cultures of *B. coli* the protein tests were due solely to the bacterial protein of the cells, and that no soluble protein was formed in the medium, as Uschinsky claimed to be the case with cultures of *B. diphtheriae* in his protein-free medium.

In comparisons of the growths in 1 per cent opsine bouillon before and after decolorization with charcoal there were rather striking irregularities, especially with the diphtheria group. Although, in general, growth was a little slower and scantier in the decolorized medium, one or two strains seemed consistently to thrive better in it. *B. diphtheriae* (No. 8) was slow in development in the decolorized medium, and did not form a film as it did in the undecolorized fluid. It was found that the decolorization resulted in only a very slight increase in the acidity of the opsine, (about +0.1), and a very small decrease in the Sörensen formalin titration for monamino acids. Animal charcoal which had been washed free of chlorides was used. On the whole, then, the undecolorized medium was rather more favorable to growth, although when agar was used the difference was very slight. It is probable that the charcoal removed something else from the medium besides the coloring matter, but no definite conclusion as to the nature of this material was reached. It was shown not to include the monamino acids.

In order to determine whether opsine could be improved as a culture medium by the addition of certain inorganic salts and carbo-hydrates the following substances were added to 2 per cent opsine and to fresh beef infusion + 1 per cent opsine.

	per cent
NaCl	0.5
KH_2PO_4	0.5
Sodium citrate	0.2
$MgSO_4$	0.2
Glucose	0.5
Glycerin	6.0

The media contained 1.5 per cent agar. Plain 1 per cent and 2 per cent opsine agar were also used. All of the media were very faintly alkaline to litmus, and inoculations were made from young agar cultures. The growths were recorded after forty-eight hours' incubation.

The growths on 2 per cent opsine alone were on the whole fully as good as those on 2 per cent opsine plus the above added substances. Every organism out of the large number tried, with the exception of one strain of pneumococcus, grew on opsine alone without beef infusion. The medium containing 1 per cent opsine + beef infusion + the above agents was a little less favorable for the gonococcus and pneumococcus (save strain 697) than 2 per cent opsine + the given mixture, but was decidedly more favorable for *B. suisepticus* and the streptococcus of equine influenza. In the case of the other organisms there was very little difference.

Experiments were also performed to compare the morphology and viability of cultures on opsine agar with those on fresh beef infusion peptone agar. These two media were prepared with reactions faintly alkaline to litmus, 1 per cent opsine, and 1 per cent peptone and 1.5 per cent agar being used. The cultures were transplanted four times on opsine agar and twice on the beef infusion peptone agar, the medium on which they were accustomed to grow. Pathogenic bacteria were incubated at 37° and saprophytic organisms at 24°. Transfers were made every two days. In general, there was little difference in the appearance and luxuriance of the growths on the two media, especially in the last transplants. Differences were distinctly apparent in the following cases: On opsine the growth of *Leptothrix* was wrinkled, while on the control medium it was larger in amount and smooth. *B. suisepticus* and *B. diphtheriae* (K.L.)

grew better on the control. On the other hand, the Hofmann bacillus showed a much better growth on opsine. Cultures of *B. prodigiosus* on opsine were of a good red tinge, but in the control tubes were colorless.

As smears from the opsine and the control cultures were made and stained side by side on the same slide, a careful comparison between the two series as to morphology and staining reaction was possible. With many organisms, the stained preparations from the two media were practically identical as to the morphology and uniformity of the bacteria, and the nature and depth of the stain. In others, however, distinct differences could be detected, which were sometimes in favor of the opsine, and again to the advantage of the control medium. Several Gram-positive organisms showed a tendency to be Gram-negative on the opsine.

In order to see if long continued residence in opsine would induce any changes, these cultures were kept at 16° for twenty-four days, then a fifth series of transplants was made to the opsine and control media respectively, and the organisms stained as before. The results on the opsine were somewhat more favorable than before, although there was no very striking or general change. Without going into much detail, it can be stated in a general way that on opsine *B. coli*, *B. pneumoniae*, *M. cholerae*, *B. avisepticus*, *B. glycobacter* (*peptolyticus*), *Streptococcus viridans*, *Proteus vulgaris* and the diphtheria group were more typical and uniform, while the control medium was the more favorable for *Bact. pullorum*, *Staph. aureus*, *B. prodigiosus*, *B. fluorescens* (*liquefaciens*) and *Proteus zenkeri*. On the whole, the pathogens, especially those that are more difficult to cultivate on ordinary media, as the diphtheria group and *Streptococcus viridans*, were more uniform and typical in morphology, and stained better, when grown on opsine than on the control beef infusion peptone medium.

In order to test the comparative viability of the organisms on the two media, the above mentioned growths (fourth transplants) were kept at 16° and subcultures made at different intervals to beef infusion and beef extract peptone agar.

Stains made at the end of twenty-four days showed that all

were pure cultures. Wherever a subculture failed to develop, the operation was repeated, heavier inoculations being made. This often changed the result.

It was found that *B. suisepticus* was alive on both media after sixteen days, but dead after twenty-four days; *B. acne* (51) was alive on both at twenty-four days, and on opsine at forty days, the control being contaminated. *Streptococcus viridans* died on the control before sixteen days, but was still viable on opsine after two months. After twenty-four days, *Leptothrix* was dead on the opsine, but living on the control, while at forty days both cultures were dead. *B. diphtheriae* (strains A, Y.M.S., and 8m), were dead on opsine at two months, forty days and twenty-four days respectively. All were alive on the control medium after two months.

B. hofmannii could not be subcultured from opsine after forty days, and from the control after two months. All the other forms were still alive on both media at the end of two months (16°).

Viability tests were also made on 1 per cent *decolorized* opsine agar, inoculated from the above fourth transplant. No controls were used.

All of the organisms grew, although in a few instances not quite as well as on the undecolorized opsine agar. After one month at 16° all of the cultures save *Leptothrix*, *B. diphtheriae* (strains a, Y.M.S., and 8m.), and *Actinomyces* were still alive. At the end of one and one-half months, the cultures of *B. suisepticus* and *B. hoffmannii* had died.

Growth, morphology and viability of menigoccocus, pneumococcus gonococcus and B. influenzae on opsine agar and on fresh beef infusion peptone agar used as control

The two strains of meningococcus employed grew about as well on opsine (1 per cent) agar as on the control peptone agar, and remained viable equally as long if not longer. Stains from the control cultures showed a great majority of peculiar involution forms of varied types, mostly thread-like filaments or spindle-

shaped forms, and very few typical diplococci. On the other hand, the stains from the opsine cultures contained very few involution forms, and were almost indistinguishable from those on blood serum. All were Gram-negative. One strain (meningococcus m) remained alive on 2 per cent opsine agar for four months at 18°. Both strains always grew on 1 per cent opsine agar in twenty-four hours, but a little less luxuriantly than on the 2 per cent medium.

Five out of the six strains of pneumococcus employed grew on 2 per cent opsine agar, four of them nearly as well as on the control medium. The fifth was carried through four transplants on 1 per cent opsine agar with rather scanty growth, and was always negative on meat extract peptone agar. In Gram stains the opsine and control cultures were both quite typical, but in the former about half of the organisms were Gram-negative, while on the control all were positive.

Viability tests on opsine agar and control meat infusion peptone agar

The meningococcus, *B. influenzae* and *B. pertussis* were carried through 12 transplants, and the pneumococcus, gonococcus and *M. catarrhalis* (a.m.) through 8 transplants on opsine. The growths on the last series were compared with those on the control medium, both being 48 hours old. The meningococcus and *B. pertussis* were grown on 1 per cent, the others on 2 per cent opsine. All of the media were faintly alkaline to litmus.

Growths at forty-eight hours

	OPSINE	CONTROL
Pneumococcus (80)	++	++
Pneumococcus (690)	++≈	++
Gonococcus (287)	≈	+≈
M. catarrhalis (a.m.)	+++	+++
B. influenzae	+≈	++
B. pertussis	++≈	++≈
Meningococcus (m)	++	++
Meningococcus (200)	++≈	++≈

The meningococcus cultures were kept at 18°, the others in the refrigerator, and subcultures were made on the control medium at various intervals. After seven days all were still alive, but after seventeen days pneumococcus no. 80 had died on both media and pneumococcus no. 690 on the opsine. At twenty-five days, pneumococcus no. 690 was still alive on the control medium, but gonococcus no. 287 had died on both media and *M. catarrhalis* on the opsine. At the end of one month pneumococcus no. 690 and *M. catarrhalis* had died on the control also.

In another lot kept at 16° for twenty-eight days, pneumococcus no. 80 was still alive on both media, pneumococcus no. 690 on opsine, but not on the control, gonococcus no. 287 on the control but not on the opsine, *M. catarrhalis* on neither, and the others on both.

On decolorized opsine the growth and viability of these organisms was distinctly less than on the undecolorized medium.

Acid-digestion products as culture media

Casein, milk albumin and edestin were the proteins chosen for the preparation of the digestion products. Casein contains all of the well-known amino acids with the exception of glycocoll and possibly cystine. In its crude form it is cheap and readily obtainable. Commercial milk albumin also is inexpensive, but it contains a limited number of amino acids, alanine, valine, leucine, aspartic acid, glutamic acid, phenylalanine, tyrosine, proline and tryptophane having been found. Edestin is a representative of the group of vegetable proteins, and contains all of the amino acids save perhaps oxyproline.

The above proteins were hydrolyzed with hydrochloric acid and the products prepared for us by Professor Lafayette B. Mendel, as follows.

Casein product A. One hundred grams of casein were heated with 400 cc. of concentrated HCl for a few hours on the water bath, under a reflux condenser. There was much charring, so the mixture was diluted with 150 cc. of water and heated over a free flame for eight hours. A large amount of humus residue was filtered off. The filtrate

was biuret-free, and gave a strong ninhydrin test for amino acids. It was subjected to prolonged evaporation on a water bath, with frequent renewal of water.

Casein product B. 40 grams of crude casein were boiled for eight hours with 200 cc. of 9 per cent HCl under a reflux condenser over a free flame. There was practically no dark residue. The liquid was biuret-free, and gave a strong ninhydrin test. It was heated in an open dish on the water bath until acid vapor ceased to come off.

Casein product C. 50 grams of the same casein used in B were boiled for eight hours with 200 cc. of 10 per cent HCl over a free flame, with a reflux condenser. The final solution was subjected to distillation, with frequent addition of water. An abundance of fatty-acid-like material came off in the distillate. This product (the distillation liquid residue) was a-biuretic, but gave a strong reaction for amino acids.

Edestin product. 40 grams of pure re-crystallized edestin were heated over a free flame with 10 per cent HCl (reflux condenser) until a biuret test was no longer given. The solution was then heated for several hours in an open dish, to remove as much hydrochloric acid as possible.

Lactalbumin product. 100 grams of crude commercial milk albumin containing about equal amounts of calcium phosphate and protein were heated two to three hours with 200 cc. of 10 per cent HCl. This treatment brought about a solution of most of the calcium phosphate. The residue of protein was filtered off and heated with 200 cc. of 10 per cent HCl until no biuret test could be obtained. The resulting solution was then subjected to prolonged heating in an open vessel, to remove as much as possible of the HCl. Finally the liquid was neutralized with NaOH for the precipitation of any remaining calcium phosphate, and filtered. Part of the filtrate was used as such; the remainder was evaporated to a sticky paste.

These products were all of a very dark brown color, but, like the opsine solution, could be easily decolorized with animal charcoal. For the sake of brevity they will hereafter be designated as "Casein A," "Casein B," "Casein C," "Edestin product" and "Lactalbumin product." The biuret test was negative in all, even by the most delicate methods. As might be expected from the severity of the treatment, all tests for tryptophane were also negative. When, however, a small amount of a weak tryptophane or peptone solution was added, a positive result was obtained, showing that the negative tests were not due to inter-

ference. Tyrosine and leucine crystals were detected in all of the solutions, on evaporation, and glutamic acid crystals in the "Lactalbumin product." Millon's test was always positive.

It was found, by evaporating to as near dryness as possible, that a medium containing 5 per cent of any of these solutions consisted of about 1 per cent solid matter. Sörensen's formalin titration for monamino acids was tried on the casein products. 5 cc. of a 5 per cent solution of "Casein A" required 4.1 cc. of $\frac{N}{20}$ NaOH to neutralize it after the addition of the neutral formalin. With "Casein B" and "Casein C" the figures were 2.9 cc. and 3.7 cc. of $\frac{N}{10}$ NaOH respectively. In an ammonia determination by Folin's method, 10 cc. of the original "Casein C" solution was found to contain 25 mgm. of N as NH_3. The other solutions were not tested.

All of these products could be decolorized and made up into culture media in the same easy and rapid way as opsine. Considerable NaOH was necessary to neutralize the media. It was found that 100 cc. of a 5 per cent "Casein C" solution required about 0.8 cc. of $\frac{N}{1}$ NaOH to make it neutral to litmus, so there was approximately 0.5 per cent of NaCl formed in the process. The other media did not differ very materially from this.

Bacterial Growth in Casein Products

In a medium of "Casein A" only, decolorized and of 10 per cent strength, 17 saprophytes and 14 pathogens, including *B. anthracis*, *B. typhi*, *M. cholerae*, *Bact. pullorum*, *B. diphtheriae*, *B. pseudo-diphtheriae* and *Streptococcus*, were grown; also two streptothrices and two moulds. When the reaction was neutral to phenolphthalein the diphtheria group grew sparsely, while with a reaction of +1 there was no growth. *B. anthracis* and *M. cholerae* also grew better on the neutral media, while *Streptococcus* showed the reverse tendency. Almost all of the other organisms grew quite well on both media. Color production was weak or entirely lacking with *B. prodigiosus*, *Staph. aureus*, *B. ruber* (*Balticus*) and *S. aurantiaca*, but good with *B. pyocyaneus* and *B. fluorescens* (*non-liquefaciens*).

In a medium of "Casein B" (5 per cent), decolorized and neutral to litmus, the same fourteen pathogens as in the last experiment were inoculated. After forty-eight hours there was no growth of *B. diphtheriae* (two strains) and of *Streptococcus*, rather scanty growth of *B. abortus* (Bang) and *B. pseudo-diphtheriae*, and good growth with all of the others. Not so many saprophytes as before were used, but all of those that were tried showed abundant growth.

For the purpose of driving off more of the HCl, some of the "Casein B" solution was evaporated over a water bath to a thick paste, and as nearly as possible to dryness. Agar was prepared from this paste in the same way as opsine agar, using 1.5 per cent of the paste. On the decolorized agar which was faintly alkaline to litmus *B. typhi* (I), *B. paratyphi A* and *B*, *Bact. pullorum*, *Spir. finkler-prior*, *Staph. albus*, *B. diphtheriae* (Y.M.S.), *B. acne* (51), *B. pertussis* and a pathogenic yeast found in tubercular sputum grew almost as well as on peptone meat extract agar. *Streptococcus* (310), *B. diphtheriae* (D), *Glycobacter peptolyticus* and *Proteus zenkeri* showed only a meagre development, and *B. diphtheriae* (K.L.) no growth at all.

These cultures were then kept at 16°C. and subcultures made at different intervals to determine their viability. After five weeks *B. diphtheriae* (Y.M.S. and K.L.), *B. acne* (51), and *B. pertussis* were found to have died. At eleven weeks all of the others were still alive.

Effect of decolorization with animal charcoal

In an experiment to determine whether the decolorizing process had any effect on the nutrient properties, "Casein B" agar was prepared as in the last experiment, one-half of the lot of medium being decolorized. Both the decolorized and undecolorized agar were inoculated at the same time from young cultures.

It was found that in general decolorization of the medium detracts little from its nutrient properties. However, *B. typhi* (10) and *Streptococcus* (urine), which grew scantily on the natural medium, failed to grow at all when the latter was decolorized, and

B. diphtheriae (a) and *B. pseudo-diphtheriae* grew a little less readily after decolorization. The undecolorized cultures were kept fifteen days longer at 16°, and then transferred again to the same medium. All but *B. typhi* (10), *M. catarrhalis* and *B. diphtheriae* (a and 8m) were still alive. The list included the meningococcus, *B. avisepticus*, *B. abortus*, *M. melitensis* and *M. cholerae* (*asiaticae*).

Effect of adding various substances to the digestion products

When 0.5 per cent Liebig's meat extract was added to "Casein B" media, the growths were quite noticeably increased. Pigment production by *Staph. aureus* and *S. aurantiaca* was also improved. On agar containing 0.5 per cent meat extract only, growths were scanty and in the case of *B. diphtheriae*, entirely lacking.

Since Vedder has claimed that starch is a valuable constituent in culture media, this was also added to "Casein B" agar to the amount of 1 per cent. No difference in the growth of the organisms could be noticed, however.

Comparisons were made between the following media, all undecolorized and containing 1.5 per cent agar; (1) "Casein C" 3 per cent, (2) "Casein C" 5 per cent, (3) "Casein C" 7 per cent and (4) "Casein C" 2.5 per cent + edestin product 2.5 per cent + lactalbumin product 2.5 per cent. On media 1 and 2 the growths were not quite so good as on medium 3. The mixture of products proved slightly superior to the other media, *Streptococcus* and *B. diphtheriae* (A) growing scantily on this, and not at all on the others. However, the mixture was not so distinctly superior as had been hoped.

Some of the more difficult organisms to cultivate were also tried out on the following media:

I. "Casein C" 8 per cent.

II. Lactalbumin product paste 2 per cent.

III. "Casein C" 2.5 per cent + lactalbumin product 2.5 per cent + edestin product 2.5 per cent.

All contained 1.5 per cent agar, were not decolorized, and were

made faintly alkaline to litmus. The results are given in the following table:

	FORTY-EIGHT HOURS		
	"Casein C" 8 per cent	Lactalbumin products 2 per cent	Medium III
Gonococcus (287)	±	−	−
Pneumococcus (M)	++	−	−
Pneumococcus (697)	−	−	−
Meningococcus (M)	+±	+±	+±
Meningococcus (200)	++	±	+±
B. pertussis	+++	++	+++
B. diphtheriae (Y. M. S.)	++	+±	+++
M. melitensis	+	−	+++
Streptococcus (urine)	±	±	++±

As was to be expected, medium II gave the poorest results. The gonococcus, pneumococcus, meningococcus and *B. pertussis* grew on at least one of these media (I), with the exception of pneumococcus 697. The same results were obtained with transplants made from these cultures to the same media.

Since tryptophane was found to be absent from all of the acid-digestion products of the proteins used, some experiments were conducted in which tryptophane was added to the different digestion products. The tryptophane exerted very little, if indeed any, influence on the nature and luxuriance of the different growths.

Comparison of growths on Witte's peptone, opsine, lactalbumin product paste, Casein C, and Casein C, edestin and lactalbumin product paste (combined)

Medium I. Witte's peptone 1 per cent, NaCl 0.5 per cent.
Medium II. Opsine 2 per cent.
Medium III. Lactalbumin product paste 2 per cent.
Medium IV. Casein C product 8 per cent.
Medium V. Casein product C 2.5 per cent + edestin product 2.5 per cent + lactalbumin product 2.5 per cent.

All of these media contained 1.5 per cent agar, and were faintly alkaline to litmus. They were all inoculated at the same time and from the same culture tube. The results are shown in the following table.

	I WITTE'S PEPTONE 1 PER CENT	II OPSINE 2 PER CENT	III LACTAL-BUMIN PRODUCT PASTE 2 PER CENT	IV "CASEIN C" 2 PER CENT	V MIXED MEDIUM
B. diphtheriae (A)	+	+++	±	±	+
B. diphtheriae (Y. M. S.)	+	+++	++	++	++±
B. diphtheriae (no. 8)	+±	++	+	++	+±
B. diphtheriae (no. 8m)	+	+++	−	−	+
B. pseudo diphtheriae	++	++±	+±	++±	++
B. abortus (B)	+	++±	+±	++	++
B. pertussis		++±			+±
M. melitensis	±	++±	+±	++	++
M. catarrhalis	+	+++	+	+±	±
Gonococcus (287)	−	+	+	+	±
Pneumococcus (m)	+	+	−	±	−
Pneumococcus (697)	+±	−	−	−	−
Meningococcus (M)	+	+	+	+	+
Meningococcus (200)	−	++	+	+	+
Streptococcus (urine)	±	+++	+	±	++
Streptococcus pyogenes (310)	+	++±		±	+
Streptococcus pyogenes (347)	+±	+++	+±	+±	++±
Streptococcus (I)	±	+		±	+
Streptococcus (IV)	±	++		±	+
Streptococcus (D)		+±		±	+
Starch bacillus (3)		+++		+±	−
Starch bacillus (6)	++±	+++		+±	+
B. acidophilus		++			−
Staphylococcus aureus (II)		+++			±
Staphylococcus aureus (III)		+++			+
Streptococcus pyogenes (VI)		++			

The two strains of a so-called "starch bacillus" were Gram—, spore-forming, starch-digesting organisms, obtained from the intestines of rats fed on a diet of cornstarch. *Staphylococcus* (II and III) and *Streptococcus* (strains I, IV, D and VI), had been freshly isolated from the milk of cows suffering from mammitis.

It is at once evident from the table that the growths on 2 per

cent opsine were strikingly more abundant than those on Witte's peptone (1 per cent). *Gonococcus* no. 287, and *Meningococcus* no. 200 grew on the opsine and not on the peptone, while the reverse was true with *Pneumococcus* no. 697. "Casein C" 8 per cent and medium V, while not as good as the 2 per cent opsine, were still superior to the 1 per cent Witte's peptone. Some organisms grew better on one of these two and some on the other. On the whole, the balance was slightly in favor of medium V. Lactalbumin product paste seemed to have, in a general way, about the same value as the peptone. In two successive transplants to the same media the cultures on "Casein C" 8 per cent and on medium V showed a decided tendency to die out. In the case of "Casein C" 8 per cent there was no growth of the streptococci nor of *Meningococcus* no. 200. On medium V the results were a little better, only two out of the five strains of streptococci dying out, in addition to *Meningococcus* 200. On the other hand, all of the cultures on the opsine medium were thriving well after five transplants on this medium.

GROWTHS IN LACTALBUMIN AND EDESTIN PRODUCTS

A few preliminary experiments demonstrated that the edestin and lactalbumin products were inferior to the casein products as culture media, so little work was done with these two. On the 5 per cent decolorized edestin product in 1.5 per cent agar, neutral to litmus, 7 pathogens and 14 saprophytes were cultivated. *Streptococcus*, *B. abortus* and the diphtheria group failed to grow, while the others did not thrive as well as on the casein products. Pigment production by *B. prodigiosus* and *B. ruber* (*balticus*) was especially striking and intense, and was also good with *Staph. aureus*, *M. cereus* (*flavus*) and *S. aurantiaca*. In the case of *B. pyocyaneus* the color was a deep blue, while cultures of *B. fluorescens* were colorless, which shows that in this medium no fluorescent pigment is produced, only the bluish pyocyanin. When transplanted to the same medium again, *B. ruber* lost its color, while after two or three transfers no change in the others was apparent. As with the other products, the addition of

Liebig's meat extract (0.5 per cent) considerably improved the medium. The color of *B. prodigiosus* cultures was not nearly as brilliant as on the edestin product alone, but the fluorescent pigment was formed by *B. fluorescens* and *B. pyocyaneus*.

It was found that the growths on the lactalbumin product paste were *much* better on 2 per cent of the paste than on 4 per cent, and *slightly* better than on 1 per cent. Too large an amount of this strongly acid paste on neutralization probably formed enough NaCl to be inhibitory. In general, this product was a better culture medium than the edestin, but inferior to the casein product.

THE GROWTH OF ANAEROBES IN THE PROTEIN-FREE DIGESTION PRODUCTS

It was surprising to find that not only *B. tetani*, *B. aerogenes* (capsulatus) and *B. botulinus*, but also the putrefactive organisms, *B. edematis* (*maligni*), *B. anthracis-symptomatici* and *B. putrificus*, grew well on the different protein-free digestion products, especially opsine.

In 2 per cent opsine *B. edematis* (*maligni*) and *B. anthracis-symptomatici* gave very heavy growths in from twenty-four to thirty-six hours. After three days of incubation spores became abundant and only a few bacilli remained. Repeated transplantation in this medium did not apparently affect the viability of these organisms.

In 2 per cent opsine plus 1 per cent glucose the growths were even more luxuriant, but the morphology of the bacilli was not the same as it was in the plain opsine. The bacilli were longer and thinner. Many were granular and stained poorly. Spore formation was indefinitely delayed, and the organisms underwent marked granulation and degeneration.

B. aerogenes (*capsulatus*) grew fairly well in 2 per cent opsine during three days' incubation. No spores were formed in plain or glucose-containing opsine. *B. putrificus* yielded a good growth in plain and in glucose opsine in five days. *B. botulinus* underwent rapid development in two to three days. Spore formation was

quite abundant. Two strains of *B. tetani* grew well in 2 per cent opsine when in association with *Staphylococcus aureus*. Spore production was quite apparent.

For comparison, plain standard bouillon and 2 per cent opsine having the same reaction were inoculated with the same organisms and incubated side by side for five days at 37°C. The results are indicated in the following table.

Growths in opsine and in bouillon; five days' incubation at 37°C.

	OPSINE 2 PER CENT	BOUILLON
B. edematis (maligni)	++	+++
B. anthracis-symptomatici (B. chauvei)	++	+++
B botulinus	+++	++
B. putrificus (A) + *Staphylococcus*	++	++

It was noticed that spore formation was always more advanced in the opsine cultures than in the bouillon. *B. putrificus* (A) was also grown with *Staphylococcus aureus* aerobically in these two media for the same length of time (five days). On the whole, when in symbiosis with *Staphylococcus aureus* it grew as well in free contact with the atmosphere as in Buchner tubes, and the morphology was about the same in both cases.

All of these anaerobes, except *B. botulinus*, were also tried in 1 per cent opsine + 1 per cent glucose, being grown in Buchner tubes for five days. All grew well. *B. edematis* and *B. anthracis-symptomatici* formed no spores, but quite typical and generally well-stained rods were numerous.

B. bulgaricus and *B. acidophilus* produced a good growth within twenty-four hours in 1 per cent opsine + 1 per cent glucose. No loss of viability was apparent after six transplantations in this medium.

SUMMARY

The protein-free enzyme-digestion product opsine serves as an excellent culture medium for both pathogenic and non-pathogenic bacteria. Practically all of the many organisms employed

grew as well or better in opsine than in Witte's peptone. The cultural characters on the two media were in general the same.

With the exception of the diphtheria group, the different organisms remained viable as long on opsine as on the standard peptone-meat-infusion medium.

A reaction slightly alkaline to litmus was found to be the best for general use. Glycerol increased the growths in alkaline but not in acid media, while glucose seemed as a rule to be somewhat detrimental. Prepared meat extract, and better still, fresh beef infusion, were distinctly favorable. Decolorization with animal charcoal slightly lessened the value of the opsine as a culture medium.

The decomposition products of casein, lactalbumin and edestin obtained by acid hydrolysis were also employed as culture media. Of these the hydrolyzed casein gave the best results, but it was inferior to the opsine. The edestin product was the least satisfactory. It is highly probable that in the hydrolysis of these proteins with hydrochloric acid the treatment is sufficient to destroy many of the intermediate decomposition products which serve so admirably as food for microorganisms.

Bacteria do not require proteins, even in minute quantities, to carry on their normal cultural development, but obtain their sustenance from less complex substances, as for example the amino acids and perhaps some of the simpler polypeptids.

In conclusion we desire to express our sincere gratitude to Prof. Lafayette B. Mendel for the preparation of the different acid-digestion products employed in this investigation, and for his hearty coöperation throughout the entire course of this work.

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THE CHARACTERISTICS OF BACTERIA OF THE COLON TYPE OCCURRING IN HUMAN FECES

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INTRODUCTION

The great value to sanitary science of a knowledge of intestinal bacteria has stimulated investigation on the colon group until an extensive literature on this subject has accumulated. The striking character of the group is its activity in fermenting carbohydrates and on this most of the attempts at classification have been based. Unfortunately, inexact methods of demonstrating and measuring complicated physiological processes have detracted from the value of some of this work. The fermentation of carbohydrates by microörganisms is evidently a much more complicated process than was formerly supposed. The intermediate and end products are subject to no little variation under the influence of the conditions of the fermentation. Conclusions based on inexact methods are necessarily open to question. This is particularly well illustrated by the history of the Smith fermentation tube, the limitations of which were better recognized by its originator than by many who have followed him in its use. At first great dependence was placed on the volume of gas evolved and the relative percentage of hydrogen and carbon dioxide produced, as determined without taking into consideration the dissolved gas. The results obtained by these methods were so erratic that the fermentation tube has come to be used merely for the determination of fermentation and the variations in the volume and ratio of gases

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have been ascribed to the uncertainties of the fermentation itself rather than to the crudeness of the method. The work of Keyes (1909) and of Keyes and Gillespie (1912) suggested the possibilities in the application of exact methods to the measurement of bacterial fermentations and the value of results so obtained in taxonomic problems.

Our own work has included a study of a collection of colon organisms from milk, one from bovine feces and one from grains. In these investigations we have endeavored to use methods which are as exact as the large number of determinations necessary for statistical treatment will reasonably permit. We first applied exact gas analysis. This has now been supplemented by more rational methods of studying acid fermentation and it is hoped that in time improvements in other cultural tests will become available.

In this paper we give the results of a study of a collection of lactose fermenting bacteria from human feces. This was undertaken primarily to establish the characters of colon cultures from the human intestine.

The failure in the past to secure satisfactory classifications on the basis of fermentation tests has been largely because the characters selected for primary divisions were not of a fundamental nature.

The gaseous fermentation appears to be a basic function of the organisms in question. Even if it is not, it furnishes a basis for primary classification which has established a considerable degree of order, not only among the results of other cultural tests but in the correlation between these tests and the sources of the cultures. We have, therefore, made exact gas determinations on all of the cultures isolated. In addition, we have determined the fermentation of various sugars and alcohols, the formation of indol from tryptophane, the formation of pigment, the liquefaction of gelatin, the carbinol or Voges-Proskauer test and the Clark and Lubs methyl red test.

The isolation of cultures. The cultures were all secured by plating fresh samples of feces. Twenty-one samples were used. These came from 18 subjects and furnished 176 cultures. The

samples came for the most part from men in normal health but included two stools from infants.

A small part of the sample taken at random with a platinum loop was thoroughly shaken in a water blank and plates made on asparagin-lactose-litmus agar (Ayers and Johnson 1915). These plates almost invariably gave colon colonies only and since it has been found that all types of colon bacilli grow readily on this medium while streptococci do not, it is very well adapted to isolation of these organisms from materials of this kind.

Several colonies from each sample were transferred to lactose broth and if gas was formed the broth was replated and a pure culture obtained. In a few cases special means were taken to favor the growth of certain types of colon organisms.

Pigment formation. Very few grain cultures of *B. coli* are entirely without pigment and many of them are decidedly chromogenic. This property is correlated with other characters and consequently is of value in classification.

Chromogenesis was determined in the human feces cultures by spreading on white paper the growth from an agar culture grown twelve days at 20° and comparing with the plates in Ridgway's Color Standards. It was found that this series was almost entirely lacking in pigment. Nearly all of the cultures gave a faint yellow color but this was so slight and showed so little variation that it was of no value. There were, however, a few exceptions to this statement.

Indol formation. Indol was determined by incubation at 30°C. in a medium containing in 1000 cc. of water 0.3 gram tryptophane, 5 gram K_2HPO_4 and 1 gram of Witte pepton. The test for indol was made by the p-dimethylamido-benzaldehyd-hydrochloric acid method. The results are summarized in table 3.

The liquefaction of gelatin. The test for gelatin liquefaction was made by spreading about 0.5 cc. of a twenty-four hour sugar free broth culture on gelatin held in a small test tube. This was incubated twenty days at 20°C. No gelatin liquefiers were found in this collection. This probably means that in these samples at least, liquefiers occurred in such small numbers that they were not isolated. There are numerous references in the

literature to the occurrence in feces of *B. cloacae* and *B. proteus* and it is likely that bacteria of this type would have been isolated if special methods had been employed for this purpose.

The fermentation of carbohydrates. The most convenient method for determining the extent of an acid fermentation is to measure the resulting hydrogen ion concentration (in terms of P_H). Some of the reasons for this have been treated in previous papers from this laboratory and need not be repeated here.

The procedure used was the following: The medium was composed of 1 per cent Witte pepton 0.5 per cent K_2HPO_4, 1 per cent of the carbohydrate or alcohol. This medium was distributed (in 10 cc. portions) in test tubes, sterilized by the intermittent method, inoculated from agar slopes and incubated for five days at 30°. The P_H values before and after fermentation were determined by the colorimetric procedure described by Clark and Lubs (1917a). A sufficient number of electrometric measurements were made to show that in the particular medium used the colorimetric P_H values were consistent and essentially correct.

The substances studied were glucose, lactose, sucrose, raffinose, melibiose, arabinose, inulin, mannitol, dulcitol, adonitol, and glycerol. Of these inulin and glycerol were commercial "c.p." samples. The other compounds were exceptionally pure samples furnished to us by Dr. Hudson of the Bureau of Chemistry. We take this opportunity to express publicly our appreciation of Dr. Hudson's kindness in affording us the rare opportunity to study the fermentation of sugars whose identification and purity is assured.

In the presence of most of these compounds the organisms within forty-eight hours either produce a vigorous acid fermentation or else very little change in the reaction of the medium. When a vigorous acid production occurs there is little doubt but that an acid fermentation of the carbohydrate is taking place. The converse proposition, that where no distinct acid production occurs the carbohydrate is not being utilized, is not so certain. There is a distinction here between the utilization of a substance and a particular mode of utilization which has not always been

kept clear and it is therefore necessary to emphasize the fact that we are in this paper dealing with the production of acid in carbohydrate media only as a cultural test.

It is now well established that the activity of cultures of *B. coli* is inhibited within a zone of hydrogen ion concentration near P_H 5.0 (Michaelis and Marcora, 1912; Clark, 1915). In any particular carbohydrate medium various cultures of *B. coli* arrive at a rather definite point and in media of various composition such points are generally not far apart (Clark, 1915). It has been clearly established that the concentration of the hydrogen ions is of far greater significance than the titratable acidity. But it can hardly be expected that any one definite hydrogen ion concentration would be found to characterize an organism in all media. If the hydrogen ion concentration exerts a constant influence upon the metabolism or growth of a culture the inhibition will become more pronounced as time passes so that a lower hydrogen ion concentration will be attained in a prolonged fermentation than in a rapid one. There are doubtless other substances formed which inhibit activity and the effect of these will be proportional to the time in which they act and to their concentration. There also may be present in a culture enzymatic actions proceeding independently of growth and general metabolism. Then too there are those opposing processes of acid production and destruction which the work of Ayers and Rupp (1917) has emphasized. In some cases the reaction observed at any time may be merely the resultant of these opposing processes.

The last condition is one which doubtless plays an important role in fermentations by the group of high ratio organisms. The tendency of these cultures to undergo a comparatively rapid reversion of reaction makes it difficult except under anaerobic conditions to supply enough sugar and to balance the buffer action of the medium so that the cultures can reach their limiting hydrogen ion concentrations. Apparently their limit is about the same as that of the *B. coli* or low gas ratio cultures.

All of these factors which enter into the determination of a reaction observed after any given period of time vary with the

particular sugar which is being fermented so that an analysis of the situation and the determination of those factors which are of fundamental importance in the fermentation of any given substance require more study than the mere determination of the P_H values attained in media indiscriminately put together. Studies of the fermentation of glucose have been sufficiently extended to enable Clark and Lubs (1915) to devise the so-called methyl red test for the differentiation of the high and low gas ratio groups. This test has been used in the present study and has been found to correlate perfectly with the gas ratios.

In testing the fermentation of other carbohydrates we have attached no particular significance to the P_H values but have used them merely to indicate whether a distinct acid fermentation has been dominant. It may be said, however, that the P_H values observed with similar organisms are not only fairly constant in media containing any easily fermented carbohydrate but they are obtained with much greater ease and convenience than the titration values formerly used in such tests.

In certain instances it has been difficult to decide whether the P_H values indicate a fermentation. This is particularly true of the fermentation of glycerol. The P_H of the medium changes very slowly and we suspect that in many instances there may have been a concomitant formation of alkali or destruction of acid which masked whatever acid formation may have occurred. A few examples showing how slow is the change in P_H in glycerol cultures are given in table 2. Results with sucrose, dulcitol and glycerol are assembled in figure 1. In dulcitol and sucrose broths there are two distinct modes separating the cultures sharply into fermenters and non-fermenters, but with glycerol there is only one distinct mode and that falls between the modes for dulcite and sucrose. It is evident that nearly all of these cultures ferment glycerol to some extent. Those cultures which show little or no change from the original reaction would possibly also be classed as fermenters if they were allowed sufficient time.

Notwithstanding the difficulty of making a sharp distinction in all cases between fermenters and non-fermenters by means of acidity determinations we prefer this to the gaseous fermentation

as measured by ordinary methods. We have observed numerous cases in which the reaction indicated an undoubted fermentation when so little gas was formed that it remained entirely dis-

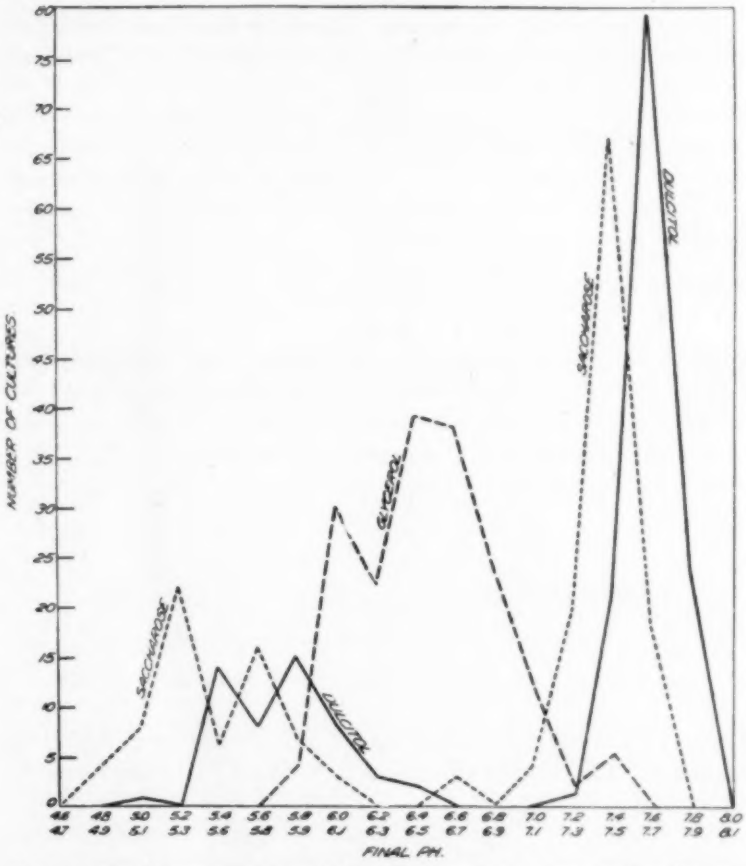

FIG 1. THE FERMENTATION OF GLYCEROL COMPARED WITH THAT OF SUCROSE AND DULCITOL

solved in the medium or appeared as a small bubble. The results of the fermentation tests are summarized in table 3.

The methyl red test. This test was made in accordance with the

procedures described by Clark and Lubs (1915) (1917b). The results given are those obtained with the old medium (Clark and Lubs, 1915). The differentiation with the new medium (Clark and Lubs, 1917b) agreed perfectly.

The Voges-Proskauer reaction. Tests for the Voges-Proskauer reaction were made with the synthetic medium of Clark and Lubs (1917b) and with pure casein and 10 per cent NaOH as reagents. The results summarized in table 3 are discussed by Clark and Lubs (1917b).

TABLE 1

Average gas volumes and gas ratios. Low ratio cultures

SOURCE	NUMBER OF CULTURES	TOTAL GAS	RATIO CO_2/H_2
Grain	8	12.4	1.06
Bovine feces	149	14.1	1.06
Human feces	130	12.7	1.05

TABLE 2

Change in P_H of glycerol medium by B. coli. Medium: 1 per cent Witte pepton, 0.5 per cent K_2HPO_4, 1 per cent glycerol. Incubation temperature, 30°. Original P_H, 7.6.

ORGANISM	THREE DAYS P_H	SEVEN DAYS P_H	TEN DAYS P_H	TWENTY-ONE DAYS P_H
vk	7.6	7.6	6.7	6.3
vr	7.5	7.3	7.2	6.7
vs	7.5		7.6	6.3
vt	7.8	6.7	6.6	6.3
vo	7.5	7.4	7.2	6.5
wd	7.6	7.6	7.6	6.7
wq	7.5	7.2	6.6	6.1
wz	7.5	7.2	6.6	5.8

Gas production. In previous studies (Rogers, Clark and Davis, 1914; Rogers, Clark and Evans, 1914; Rogers, Clark and Evans, 1915) it was found that accurate determination of the gas volumes and gas ratios produced in the anaerobic fermentation of glucose furnished most valuable data. In the present study we have employed the methods for the isolation and analysis of the gas which were described in previous papers. Essentially, the

procedure consists in growing the bacteria in 10 cc. of 1 per cent Witte pepton, 0.5 per cent K_2HPO_4, 1 per cent glucose, held in evacuated sealed bulbs for seven days at 30°, collecting the gas by evacuation with mercury pumps and analyzing the gas over mercury.

The average gas volume and average gas ratio for the 131 low ratio cultures from human feces are shown in table 1 together with the averages found in the grain and bovine feces series. It

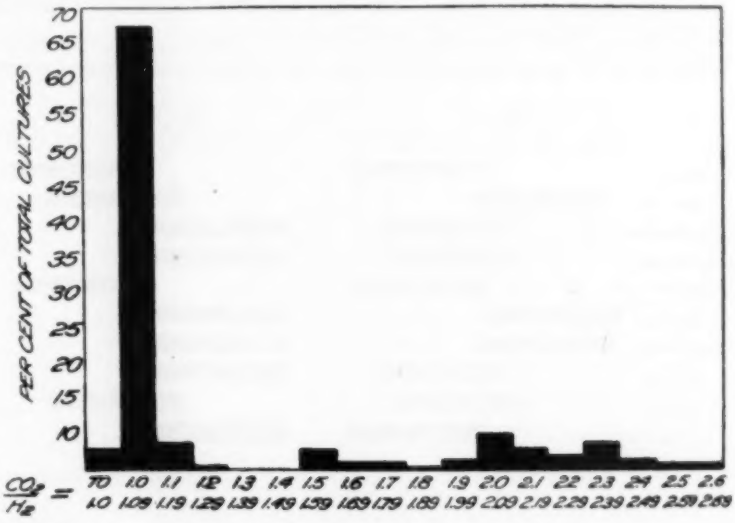

FIG. 2. DISTRIBUTION OF CULTURES ACCORDING TO $CO_2 : H_2$ RATIO

may be noted that the average ratio 1.06 is identical with that found by Keyes and Gillespie when their organisms were cultivated in a synthetic medium.

To bring out more clearly the sharp distinction between the high ratio and low ratio groups the distribution of cultures by gas ratios is represented graphically in figure 2.

The significant characters of the fecal cultures. It is shown in figure 2 that, on the basis of the frequency of occurrence of certain carbon dioxide-hydrogen ratios the cultures are separated into the two distinct groups already observed in cultures from milk,

from bovine feces and from grains. It should not be assumed, however, that the relative numbers of cultures as shown in this chart are indicative of the proportions in which they occur in the intestines. In bovine feces the high ratio or *B. aerogenes* group occurred so rarely that we obtained only one culture of this type in the 150 isolated. In human feces the proportion of high ratio cultures is usually greater but varies with the individual and perhaps with certain physiological conditions. In only 3 samples were *B. aerogenes* cultures obtained by direct plating. In one of these, one of seven was *B. aerogenes*, in another, 3 of 6 and in the third, of 52 colonies subjected to the methyl red test 31 were of the

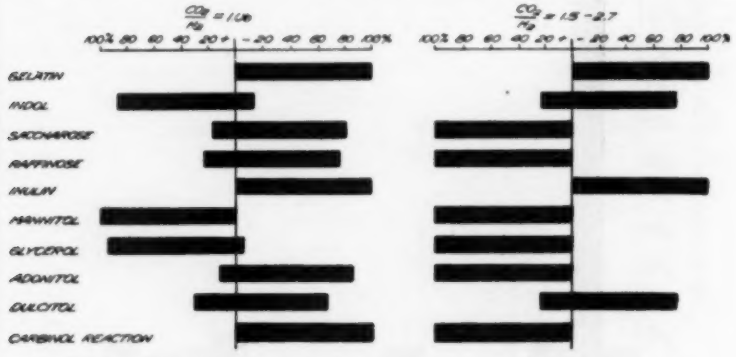

FIG. 3. PHYSIOLOGICAL CHARACTERS OF THE B. COLI-AEROGENES TYPES

B. aerogenes type. All other high ratio cultures were obtained by special methods. Mr. Ayers called our attention to the fact that when sterile milk was infected with bovine feces and incubated at 20° the high ratio type predominated at the time of curdling. This holds true also for human feces and the greater part of our high ratio cultures was obtained by plating milk which had been curdled by inoculating with feces and holding at 20°C.

The predominance of the *B. aerogenes* type under these conditions is probably explained by the observation, made in the course of another investigation, that the *B. aerogenes* type is less retarded by low temperatures than is the *B. coli* or low ratio type.

The fermentation reactions of the two types of cultures are

TABLE 3

Showing percentage of positive physiological reactions

	TOTAL CULTURES		INDOL	CARDINOL REACTION	SUCROSE	RAFFINOSE	INULIN	MANNITOL	GLYCEROL	DULCITOL	ADONITOL
Low ratio...	131	Number +	127	0	23	31	0	130	124	41	17
		Per cent +	96.95	0	17.56	23.66	0	99.23	94.66	31.78	12.98
High ratio...	46	Number +	10	46	46	46	0	46	46	10	46
		Per cent +	21.74	100	100	100	0	100	100	21.74	100

TABLE 4

Per cent of positive reactions obtained with cultures from various sources

GROUP	SOURCE OF CULTURES	NUMBER OF CULTURES	INDOL	SUCROSE	RAFFINOSE	INULIN	MANNITOL	GLYCEROL	ADONITOL	DULCITOL
			per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Low ratio (coli)	Milk	58	87.90	39.70	43.10			56.90	15.80	50.00
	Bovine feces	149	99.30	59.10	62.40	0	100.00	97.30	20.80	68.20
	Human feces	131	96.95	17.56	23.66	0	99.23	94.66	12.98	31.78
	Grains	8	50.00	25.00	75.00	12.5	100.00	25.00	37.50	50.00
	Feces*	306	98.02	46.08		0			9.48	74.18
	Feces†	117	95.72	41.88	51.28			75.82		53.85
High ratio (aerogenes).	Milk	56	60.70	96.40	91.00			18.50		26.80
	Human feces	46	21.74	100.00	100.00	0	100.00	100.00	100.00	21.74
	Grain	111	7.20	88.29	85.58	10.81	20.72	56.76	12.61	16.21
	Feces*	11	0	100.00		0			100.00	27.27

* MacConkey, 1909. MacConkey's results are recalculated assuming that all V and P positive cultures are *B. aerogenes* and V and P negative cultures are *B. coli*.

† Levine (1916). Includes cultures from horse, sheep, cow and man.

shown in table 3 and figure 3. The correlation between the nature of the fermentation as expressed by the resulting gases and the ability to produce acid from certain substances agrees very closely with that which we have found in the colon cultures from other sources and with the correlations found by other investigators.

This agreement is shown in table 4 which includes our own

data, that of some 300 cultures whose characters have been tabulated by MacConkey and the data of 117 cultures reported by Levine. The results obtained by Levine show that it is safe to separate MacConkey's cultures into the high and low-ratio groups by means of the Voges and Proskauer test. If we exclude the eight grain cultures, the agreement among the low ratio cultures is as close as could be expected after making proper allowance for differences in methods. The relatively low percentage of indol formers among the milk cultures is probably due to the use of a method which was later found to give low results.

It will be noted that there was an appreciably higher number

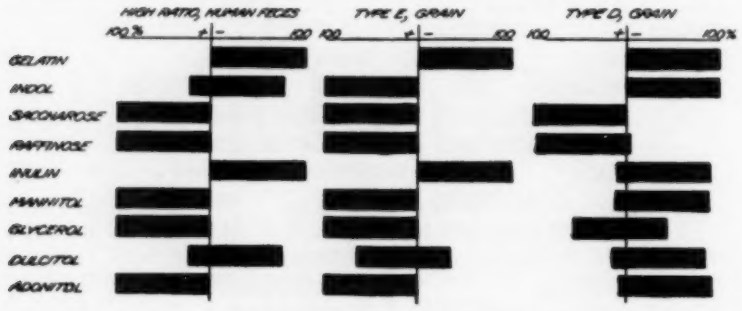

FIG. 4. A COMPARISON OF THE HIGH RATIO CULTURES FROM FECES WITH TWO TYPES FROM GRAINS

of cultures fermenting sucrose, raffinose and dulcitol among the bovine feces cultures than among those from human feces. MacConkey's cultures, which in this respect are intermediate between the human and bovine cultures, include 138 from feces of various animals.

While Levine's results as to the relative number of sucrose fermenters in feces from various animals are not conclusive on account of the comparatively small number from each source, he found a decidedly higher percentage of sucrose and raffinose fermenters among the cultures from the lower animals than among those from man. The percentages for sucrose fermentation ranged from 32.3 per cent for cultures from pigs to 95.5 per cent for the sheep as against only 12 per cent for man.

Browne (1915) found that about 11 per cent of colon cultures from human stools fermented sucrose while about 40 per cent fermented dulcitol. These results are in fairly close agreement with our own.

When we turn to the high ratio or *B. aerogenes* group we find a more complex situation. The non-liquefying high-ratio grain cultures were easily separable into three distinct types. For purposes of comparison two of these are arranged with the high ratio fecal group in figure 4. Type E, in which were included only 8 cultures, presents a picture almost identical with that shown by the diagram for the high ratio fecal group. There was a higher percentage of indol formers among the grain cultures and a slightly larger proportion of dulcitol fermenters; otherwise the agreement is perfect and it may be assumed that the 8 grain cultures were originally of fecal origin.

The diagram for type D, which included 90 cultures, shows the typical *B. aerogenes* culture found on grains. While it agrees with the high ratio human feces cultures in certain characteristics it is sharply differentiated from them by its feeble action on the alcohols, particularly on adonitol. The *B. aerogenes* cultures from feces without exception ferment this alcohol readily while with the exception of those classified as of fecal origin only 6 of 103 of the *B. aerogenes* cultures from grains were able to utilize this substance.

These results indicate that there is reason for separating the high ratio type found in feces from the high ratio type found on grain as shown by type D in figure 4. The difference in ability to utilize adonitol is distinctive and if the separation is a logical one it should be of value to water bacteriologists.

In a previous paper (Rogers, Clark and Davis, 1914) it was shown that the low gas ratio if not modified by oxidations of the hydrogen remains constant under a variety of conditions which permit wide variations in the high gas ratios. This together with other considerations led to the conclusion that the carbon dioxid and the hydrogen liberated by *B. coli* are intimately connected. The establishment of so constant and simple a ratio between two products furnishes a most reliable sort of cultural characteristic

and justifies our setting apart the low ratio organisms as distinct from the more heterogeneous collection of high ratio organisms. Whether or not this will ultimately prove to be sound judgment the fact remains that it has been of great empirical value. But we have not been content with using this basis of classification alone. If it is a proper basis for a primary classification, there should appear decided correlations between the gas ratios and other cultural tests. As our studies have progressed, evidences of such correlations have become stronger and are becoming more clearly established as various tests become more rational and

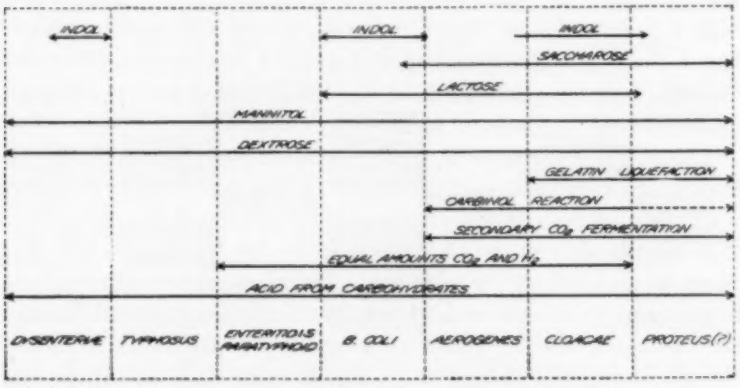

FIG. 5. GRAPHIC REPRESENTATION OF THE SALIENT CHARACTERS OF THE TYPHOID COLON, AEROGENES GROUP

exact. Hulton (1916) in discussing our results says: "In their system of classification, the gas ratio formed the only basis of differentiation; members classed within a single group display marked differences in powers of liquefaction, indol-production, and motility, but all agree in gas ratios." In view of the fact that tests of indol production, gelatin liquefaction and motility have not yet been placed upon an exact basis and because the gas ratios correlate perfectly with the methyl red and Voges-Proskauer tests in the present series, we should perhaps be willing to have it understood that the gas ratios formed the only basis of primary classification. This, however, would hardly be a correct

statement of our views since we have shown by biometric treatment correlations among other cultural characteristics which are quite as good as those obtained in other schemes of classification.

Kligler (1914), as the result of a study of 80 cultures in the collection of the American Museum of Natural History, proposed a classification based upon the ability to ferment certain sugars and alcohols. This was a step in advance in that it was based on correlated characters. We are not ready to admit, however, that the fermentation of polysaccharides is in itself a sufficiently fundamental character to be used for major divisions of a large group.

Levine (1916b) has proposed a classification of the colon-aerogenes group in which particular attention is given to those cultures which are included in figure 5 as *B. coli*. Six species are made of this group, one of which includes two varieties. The separations are made on sucrose fermentation, motility and the fermentation of salicin, dulcitol and glycerol. We have always considered that *B. coli* as represented in figure 5 is comparatively a homogenous group and that the differences indicated by variations in sugar fermentations are not of specific rank. On the other hand, we have looked upon *B. aerogenes*, which Levine includes as a single species, as a heterogenous group much in need of further division.

The greatest value of definite knowledge of the characteristics of a class of organisms lies in the ability which it gives us to arrange these organisms in orderly groups. Ever since the beginning of the science bacteriologists have attempted to do this and as the knowledge of bacteria has advanced new systems of classification have been proposed, many of them differing radically from their predecessors.

The cumulative result of these attempts to create order has brought such confusion that we have hesitated to apply our results to any system of classification for the colon-aerogenes group. We feel, however, that the data which have accumulated in the last few years have sufficient value to warrant us in outlining their possible application to taxonomic problems. While there is no generally accepted scheme of classification for the typhoid-colon

aerogenes group there are certain salient points which nearly all bacteriologists will agree may be accepted as separating the group into rather loosely defined subgroups or species. These may be outlined briefly as follows:

Characters common to the group—short, thick, gram negative bacillus, tendency to ferment carbohydrates to some degree; normal habitat, intestines of warm-blooded animals

Dysenteric group	Acid from glucose and sometimes from mannitol and sucrose. Indol, ±. Carbinol reaction—Characteristic serologic and pathogenic properties.
Typhoid group	Acid from glucose and mannitol. Indol and carbinol reaction −. Characteristic serologic and pathogenic properties.
Paratyphoid, enteritidis group	Acid and gas from glucose, mannitol and dulcitol. Indol and carbinol reaction negative. Characteristic serologic and pathogenic properties.
B. coli group	Acid and gas from glucose, lactose, mannitol and sometimes sucrose and dulcitol. Volume of gas relatively small. Indol usually positive, carbinol reaction usually negative.
B. aerogenes group	Acid and gas from glucose, lactose, sucrose and usually from mannitol, but not from dulcitol. Volume of gas greater than *B. coli*. Indol − and carbinol reaction ±.
B. cloacae group	Characters very similar to aerogenes and in addition gelatin is liquefied.
Proteus group	Liquefies gelatin and under certain conditions forms characteristic "swarming" colonies. Acid and gas from glucose but lactose is not fermented.

A more definite classification of the colon-aerogenes group is offered by the MacConkey arrangement which was adopted by the laboratory section of the American Public Health Association. This is purely an arbitrary classification based on the possible mathematical combinations of positive and negative reactions obtained with dulcitol and sucrose. It has no logical basis or practical value.

The data concerning the fundamental characteristics of the several groups in this large family of bacteria is without doubt too meagre at present for the establishment of a system of classifica-

tion which will have permanent value. There is, however, some justification in proposing a scheme of classification if it possesses those elements of plausibility and suggestiveness which render working hypotheses valuable assets in research. We now suggest such a scheme which is based primarily upon a hypothesis proposed in the first paper of this series (Rogers, Clark and Davis, 1914).

The internal mechanism by which an organism converts its food into energy and cell material is conceived to be more deep seated and less variable than the processes by which a particular substance is prepared for utilization. It is generally assumed that substances like sucrose must be hydrolized before the constituent glucose or fructose can be utilized. While definite evidence of this is lacking we may assume it to be true. It is then conceivable that since the inverting enzyme has only a preparatory function it may be subject to some variation as the food supply of the bacteria changes while the processes through which the ultimate utilization of the carbohydrate is brought about remain constant. The nature of the inverting enzymes is shown by the particular polysaccharides utilized while the processes which produce the final decomposition are indicated by the end products of the fermentation.

Such a conception assigns a position of secondary importance to certain sugar fermentations and leads to a search for the significant aspects in the ultimate metabolism. In the colon-aerogenes group an indication of one special mechanism of fermentation is found in the varying amount and nature of the gases produced.

As previously suggested (Rogers, Clark and Davis, 1914) the reaction by which carbon dioxid and hydrogen are liberated in equal volumes may be common to the high and low ratio organisms and the high gas ratio may result from an additional reaction in which carbon dioxid alone is set free. It is suggestive to note that we now have one group which produces equal volumes of carbon dioxid and hydrogen, another which produces an excess of carbon dioxid and a third which produces carbon dioxid only and that all three groups appear to be generically

related in so far as a general similarity in their morphology, relation to external conditions and their metabolic activities betokens such a relationship.

Upon such a basis, which is of course hypothetical, there can be constructed the provisional scheme of classification shown in figure 5. This scheme recognizes in all members of the group an ability to bring about some sort of fermentation of the simplest carbohydrates. In what may be considered the lower members of the group this fermentation is limited to the formation of acids from simple sugars such as glucose. The four higher groups beginning with *B. enteritidis* possess the ability to institute a fermentation in which carbon dioxide and hydrogen are formed in equal volumes. , The *B. aerogenes* and *B. cloacae* groups are differentiated still further by a secondary fermentation producing additional carbon dioxide. With this is correlated the somewhat obscure carbinol reaction and in the case of *B. cloacae* the secretion of proteolytic enzymes. The position of the so-called *B. proteus* group in this diagram is questionable. We have designated as *B. proteus* those organisms which produce no hydrogen. This is not compatible with the characteristics of several cultures which others have furnished to us as *B. proteus* but it is based on data obtained with some of these *B. proteus* cultures from other laboratories and on data obtained with seven cultures in our collection from grain. All of these seven cultures liquefied gelatin and six failed to ferment lactose. They would ordinarily have been classed as *B. proteus*, but whatever name is used, such a group exists and is of relatively common occurrence.

Inasmuch as all of our *B. proteus* cultures ferment sucrose they have the general characteristics which would be possessed by a *B. cloacae* culture in which the fermentation producing equal volumes of carbon dioxide and hydrogen had been suppressed.

This diagram, we believe, represents the probable evolution from the highly organized *B. cloacae* group, amply qualified to live an independent existence, to the parasitic pathogenic types. This hypothesis is in accord with the reported distribution of the different types. *B. cloacae* is common in soil and water while *B. aerogenes*, its close relative, was found by us very commonly

on grains and in water but only occasionally in feces. *B. coli*, on the other hand, is the principal organism found in the intestines and does not thrive in natural conditions outside of the animal body. All of the evidence available indicates that the enteritidis, typhoid and dysenteric groups do not live normally outside of the animal body. The proteus group is possibly an offshoot of *B. cloacae* in which a part of the fermentative ability has been lost.

No biological species or genus has sharply defined limits and it should not be assumed that the physiological characters are restricted in the precise manner indicated in this diagram. It is commonly assumed, for instance, that all members of the colon-aerogenes group ferment lactose but it is very improbable that a sharp distinction could be drawn in this way without excluding cultures which by all other characters belong in this category. While this distinction is habitually made in water bacteriology, it is generally recognized that the exclusion of cultures, which, while otherwise resembling colon, do not ferment lactose, is a concession to the demand for rapid results justified by the fact that it does not introduce a serious error.

Clemesha (1912) speaks of a colon organism fermenting glucose but not lactose as making up 4 or 5 per cent of the flora of the intestines and says that lakes in India exposed for some time to sunlight contained more of this type than any other. Our own collection from human feces was made on the basis of fermentation of lactose in conformity with the usual practice of American sanitarians. It included, however, one culture which fermented glucose but not lactose. This did not differ otherwise from the typical low ratio culture.

It may be argued that lactose is a unique carbohydrate in that it is distinctly an animal sugar and that its fermentation or non-fermentation is of more fundamental significance than the fermentation of a distinctly plant sugar. We fail to see any logic whatever in such an argument. It is comparable with connecting fermentability with optical rotation, neglecting the more weighty matters of configuration and linkage within the sugar group. It may be significant to note that of 177 cultures which

we have studied in the present series all but one ferment both lactose, a sugar of animal origin, and melibiose, a plant sugar closely related to lactose. The one culture which failed to ferment lactose failed to ferment melibiose.

In this scheme of classification the positions assigned to the lower groups are determined in large measure by the gas formation. While the data found in the literature are for the most part unreliable and the results which we have obtained are meagre, the following determinations are significant.

Cultures of *B. paratyphi*, *B. enteritidis* and *B. dysenteriae* from the American Museum were grown at 37°C. on the medium used in the gas determinations with colon cultures and the gas volume and ratios were determined by the accurate method previously described.

The results are as follows.

ORGANISM	TOTAL GAS	RATIO CO_2/H_2
B. paratyphi "A." American Museum no. 294	7.09	1.00
B. paratyphi "A." American Museum no. 16	13.83	1.03
B. paratyphi "B." American Museum no. 323	8.60	1.06
B. paratyphi "B." American Museum no. 22	10.08	1.01
B. enteritidis American Museum no. 18	12.24	1.03
B. dysenteriae (Kruse) American Museum no. 121	Trace	Not analyzed
B. dysenteriae (Shiga) American Museum no. 197	Trace	
B. dysenteriae (Flexner) American Museum no. 110	Trace	

Keyes and Gillespie (1912) have reported hydrogen and carbon dioxide as products of the activity of *B. typhi*. This might be considered to invalidate the position given to *B. typhi* in figure 5. However, Keyes and Gillespie obtained only about 0.2 cc. total gas per 10 cc. with *B. typhi* and the gas ratio CO_2/H_2 was large and inconstant.

Theoretically it is of course unnecessary to assume that carbon dioxide is a constant product of the metabolism of bacteria, yet experimentally there are evidences that small amounts of this gas are formed by certain "non-gas producing" bacteria. This is neglected in figure 5 as an unessential consideration.

SUMMARY

A collection of 177 cultures of the colon-aerogenes type was made from human feces.

These were examined for pigment formation, indol production, liquefaction of gelatin, the acid fermentation of glucose, lactose, sucrose, raffinose, melibiose, arabinose, inulin, mannitol, dulcitol, adonitol, and glycerol, the methyl red test, the carbinol or Voges-Proskauer reaction and the anaerobic production of gas from glucose as determined by exact methods.

Of the 177 cultures studied, 131 gave a CO_2/H_2 ratio of approximately 1.06. The remaining 46 cultures gave ratios varying from 1.5 to 2.7.

The methyl red tests correlated perfectly with the gas ratios.

None of these cultures fermented inulin but practically all fermented mannitol and glycerol. Nearly all of the low ratio cultures formed indol but all were negative to the carbinol reaction. A relatively small percentage fermented sucrose, raffinose, dulcitol and adonitol.

The high ratio cultures included a small percentage of indol producers but all gave a positive carbinol reaction. All of them fermented sucrose, raffinose, and adonitol. Only a small number fermented dulcitol.

The high ratio group agreed very closely with a group represented by a small number of cultures isolated from grains and described in an earlier paper. The greater number of the grain cultures differed from the fecal cultures in their ability to ferment alcohols, particularly adonitol.

A tentative scheme of classification for the typhoid colon group is presented in which the primary divisions are based on the nature of the metabolism as indicated by the gases evolved.

The gas analysis of a few available cultures of the paratyphoid group gave a gas volume and ratio agreeing with that of the colon type.

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A STATISTICAL CLASSIFICATION OF THE COLON-CLOACAE GROUP

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It is now firmly established that the end products of metabolism, as well as morphological differences, are reliable and convenient indices for differentiation of bacterial species and varieties. In the group of coli-like bacteria, particular attention has been paid to acid and gas production fron various fermentable substances.

Theobald Smith (1893) observed that some *B. coli* ferment sucrose and therefore recognized two forms.

Durham (1901) suggested the name *B. coli-communior* for the sucrose fermenting variety and characterized *B. lactis-aerogenes* as a polysaccharid fractor.

MacConkey (1905 and 1909), whose classification has been most widely employed, subdivided upon gas formation from sucrose and then from dulcitol thus giving 4 main types generally known as the *B. acidi-lactici* type (sucrose negative, dulcitol negative); the *B. communis* type (sucrose negative dulcitol positive); the *B. communior* type (sucrose positive, dulcitol positive), and the *B. aerogenes* type (sucrose positive, dulcitol negative). Under each type are recorded a number of varieties according to gelatin liquefaction, indol production, the Voges-Proskauer reaction, motility, and fermentation of inulin, adonitol, etc.

Very much along the lines of the MacConkey classification is that of Bergey and Deehan (1908). They employed 8 characters —fermentation of sucrose, dulcitol, adonitol, and inulin; gelatin liquefaction, indol production, motility and Voges-Proskauer reaction—and from a consideration of all possible combinations

between these characters recognized the possible existence of 256 varieties of *B. coli*.

The grouping of Jackson (1911) which was accepted by the American Public Health Association and included in the standard methods for 1912, is very similar to that of MacConkey, but here preference is given to dulcitol over sucrose for the primary division. Each of the 4 groups thus formed is subdivided further on raffinose and mannitol, and then on motility, indol, reduction of nitrates, and gelatin liquefaction.

A very serious objection to the classifications of MacConkey, Bergey and Deehan, and Jackson, is their extreme flexibility. As the number of fermentable substances, or other characters observed, increases, the number of "varieties" increases geometrically approaching infinity. The number of "varieties" is given by the formula 2^n where 'n' is the number of characters studied. Thus with 8 characters there are 256 possible combinations; this number rises to 1024 with 10 characters and to 65,536 when 16 characters are observed. The absurdity of regarding each character as of similar and equal differential value is thus evident. In the more recent studies the principle of the correlation of characters has been emphasized.

Howe (1912), from a statistical study of 630 strains of *B. coli* isolated from human feces, concludes that dulcitol, indol production, nitrate reduction, etc., are not correlated with each other nor with vigor of growth, and he therefore recognizes only the sucrose positive *B. communior* and sucrose negative *B. communis*.

Rogers and his associates, (1914–1916) studied a large number of coli-like forms from milk, grains, and bovine feces, and conclude that two distinct groups may be recognized on the basis of the accurately determined gas ratio—the low ratio *B. communis-B. communior* group and the high ratio *B. aerogenes-B. acidi-lactici* group. There is no doubt that *B. communis* and *B. communior* are low ratio strains and *B. aerogenes* of the high ratio group but the inclusion of *B. acidi-lactici* with *B. aerogenes* does not seem justified, and I believe that further studies will place it definitely with the low ratio strains.

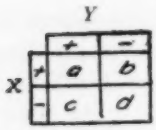
Kligler (1915) suggests that salicin be substituted for dulcitol, in subdividing coli-like bacteria, pointing out that salicin fermentation correlates better with the Voges-Proskauer reaction than does dulcitol decomposition. He thus recognizes a sucrose negative, salicin negative group (*B. acid-lactici*); sucrose negative, salicin positive group (*B. communis*); sucrose positive, salicin negative group (*B. communior*) and sucrose positive, salicin positive (*B. aerogenes*). *B. cloacae* is differentiated from *B. aerogenes* by its inability to ferment glycerol.

The characterization of *B. communior* as salicin negative is probably untenable. The term *B. coli-communior* was first employed by Durham to describe a variety of *B. coli* which fermented sucrose and which was motile. Later Ford recognized it as a species *B. communior*. Such organisms usually ferment salicin as will be shown later in this paper.

Where the principle of correlation has been employed the best correlated character has apparently been picked out by inspection of the data. Inspection is a tedious and difficult procedure, entirely inapplicable where the number of characters considered is large, and it does not permit of a concise statement of the degree of correlation which exists between different reactions. Considerable information in an abstract, concise, and workable, form may however be obtained from a study of the coefficients of correlation.

THE COEFFICIENT OF CORRELATION

Where we are concerned merely with the presence or absence of characters the coefficient of correlation between any two characters may be easily determined. Suppose that it is desired to know if the characters X and Y are correlated and that a study of a number of organisms showed that 'a' cultures are positive for both X and Y; 'b' organisms positive for X but negative for Y, 'c' cultures are negative for X and positive for Y; and 'd' strains are negative for both X and Y. The distribution of the organisms is first tabulated as shown below.



The degree of association, or the coefficient of correlation, is then expressed, according to Yule, by the formula

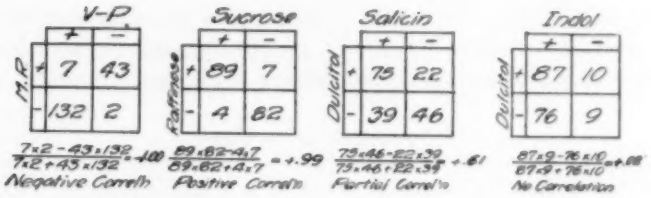
$$\frac{ad - bc}{ad + bc} \tag{1}$$

If 'ad' is equal to 'bc' the coefficient becomes $\frac{0}{ad + bc}$ or 0; which indicates that there is no correlation whatever. If either 'b' or 'c' is zero the formula becomes $\frac{ad}{ad} = 1$; indicating a perfect positive correlation. If 'a' or 'd' is zero then we have $\frac{-bc}{bc} = -1$; showing a perfect negative correlation. It should be observed that an absolute positive correlation exists in reality only if both 'b' and 'c' are zero and an absolute negative correlation when both 'a' and 'd' are zero. In order to avoid coefficients of 1 or -1 where only one group—'a', 'b,' 'c,' or 'd'—is zero. Yule gives the formula

$$\frac{a\,(a+b+c+d) - (a+c)\,(a+b)}{\sqrt{(a+c)\;(b+d)\;(a+b)\;(c+d)}} \tag{2}$$

In practice, however, a few strains are almost always found in each of the four groups and Yule suggests the use of the simpler formula (1). Some caution should therefore be employed in interpreting coefficients of 1 or -1.

For this study it was assumed that if the coefficient between two characters is numerically greater than 0.5 they may be regarded as correlated, but if less than 0.3 there is probably no association. A few examples of correlation coefficients actually obtained in the course of this study are given to illustrate the method of calculation.



The principle of correlation should not be applied indiscriminately to collections of data for systematic purposes. Certain characters and properties have been universally accepted as reliable and appropriate for bacterial differentiation; thus, staining reactions such as the Gram and acid fast stains; spore formation, aerobiosis and anaerobiosis, hardly need to be bolstered up by correlation with other characters to justify their taxonomic value. On the other hand the significance of such characters as motility, indol production, and fermentation of certain substances, is still debatable.

Motility is regarded by many as a highly variable property. Perhaps it is in reality a reliable morphological difference. Certainly if it could be shown that this character goes hand in hand with several others, more reliance and attention should and would be given to motility. The same is true of the indol test. In dealing with gas formation from carbohydrates, alcohols, or polysaccharids, the question naturally arises as to which substance should be given preference for subdivision, or whether all are to be considered of equal taxonomic value. The lack of a criterion for determining the most significant fermentable substances has led to considerable confusion. It has already been pointed out how subdivision on every character studied results in an infinite number of varieties. Where we are dealing with a number of characters each of which is assumed to be of equal taxonomic significance, it would certainly be desirable and advantageous to subdivide on that character which gives the greatest amount of information as to the manner in which the resulting subgroups react with respect to other characters. It is under such circumstances that the principle of correlation of characters may be legitimately, conveniently, and advantage-

ously employed. It may be recalled that the differentiation of the colon-intermediate-typhoid group on glucose and lactose fermentation is strikingly correlated with pathogenicity.

STATISTICAL STUDY

In the following pages is evolved a classification of coli-like bacteria based primarily upon correlated characters. The study is made upon 333 organisms obtained from soil, sewage and the feces of man, horse, sheep, pig and cow.

The characters considered are the methyl-red and Voges-Proskauer reactions, indol production, motility, gelatin liquefaction and gas formation from sucrose, raffinose, dulcitol, glycerol, salicin, dextrin, inulin and corn starch. Other fermentable substances—lactose, maltose, galactose and mannitol—were also observed but as these substances were all attacked with gas formation they need not be considered.

The investigations of Theobald Smith, Hardin, Rogers and others indicate distinctly that the Voges-Proskauer positive or methyl-red negative strains are so different from the Voges-Proskauer negative, or methyl-red positive organisms with respect to the end products of carbohydrate fermentation that subdivision upon these characters seems justified. Two groups are therefore recognized, the methyl-red positive, Voges-Proskauer negative or *B. coli group* and the methyl-red negative, Voges-Proskauer positive or *B. aerogenes-B. cloacae* group.

METHOD OF STUDY

The organisms in each of the two groups are first tabulated as in table 1 in order to facilitate the calculation of the correlation coefficients which are then determined for each pair of characters and recorded as indicated in table 1A. In choosing between any two characters, that one which gives the highest coefficient of correlation with the greatest number of other characters is selected for subdivision. For the resulting subgroups new correlation tables are prepared and subdivision again made as above. A point is very quickly reached where further subdivision upon

correlated characters is no longer feasible. These groups are regarded as species and to each is assigned, as far as possible, the name of the MacConkey variety which it most resembles.

TABLE 1

Showing correlation of characters among 151 strains of the Aerogenes-cloacae group

		Gelatine		Motility		Indol		Sucrose		Raffinose		Dulcitol		Glycerol		Salicin		Dextrin		Inulin*		Starch	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Gelatine	+	83		81	2	13	70	83		79	4	11	72	7	76	82	1	27	56	4	79	5	78
	−		68	8	60	33	35	65	3	66	2	34	34	62	6	67	1	63	5	18	41	60	8
Motility	+	81	8	89		15	74	86	3	83	6	14	75	8	81	87	2	28	61	4	84	4	85
	−	2	60		62	31	31	62		62		31	31	61	1	62		62		18	36	61	1
Indol	+	13	33	15	31	46		46		46		24	22	37	9	46		39	7	9	31	33	13
	−	70	39	74	31		103	102	3	99	6	21	84	32	73	103	2	51	54	13	89	32	73
Sucrose	+	83	65	86	62	46	102	148		144	4	44	104	69	79	146	2	90	58	22	117	65	83
	−		3	3			3		3	1	2	1	2		3	3			3		3		3
Raffinose	+	79	66	83	62	46	99	144	1	145		44	101	68	77	143	2	88	57	21	115	65	80
	−	4	2	6			6	4	2		6	1	5	1	5	6		2	4	1	5		6
Dulcitol	+	11	34	14	31	24	21	44	1	44	1	45		38	7	45		37	8	11	22	33	12
	−	72	34	75	31	22	84	104	2	101	5		106	31	75	104	2	53	53	11	89	32	74
Glycerol	+	7	62	8	61	37	32	69		68	1	38	31	69		69		66	3	20	41	63	6
	−	76	6	81	1	9	73	79	3	77	5	7	73		82	80	2	24	58	2	79	2	80
Salicin	+	82	67	87	62	46	103	146	3	145	6	45	104	69	80	149		89	60	22	119	65	84
	−	1	1	2			2	2		2			2		2		2	1	1		1		2
Dextrin	+	27	63	28	62	39	51	90		88	2	37	53	66	24	89	1	90		22	60	63	25
	−	56	5	61		7	54	58	3	57	4	8	53	3	58	60	1		61		60		61
Inulin*	+	4	18	4	18	9	13	22		21	1	10	11	20	2	22		22		22		21	1
	−	79	41	84	36	31	89	117	3	115	3	22	89	41	79	119	1	60	60		120	36	84
Starch	+	5	60	4	61	33	32	65		65		33	32	63	2	65		65		21	36	65	
	−	78	8	85	1	13	73	83	3	80	6	12	74	6	80	84	2	25	61	1	84		86

* Nine strains not tested in inulin.

THE AEROGENES-CLOACAE GROUP

In the *B. aerogenes-B. cloacae* group are included all strains which gave the Voges-Proskauer reaction—(practically always alkaline to methyl-red) and 10 cultures which fermented starch with gas formation but did not react typically for the Voges-Proskauer nor methyl-red tests. There are 151 organisms in the group, 9 of which were obtained from sewage and the rest, 142, from soil.

The distributions of the strains with respect to gelatin liquefaction, motility, indol and gas formation from sucrose, raffinose, dulcitol, glycerol, salicin, dextrin, inulin and starch are shown in table 1. Mannitol, maltose, lactose and galactose were always fermented and are therefore not included.

Gelatin was liquefied by 83 (55 per cent) (observed for thirty-four days at 20°C.); 89 (59 per cent) were motile; 46 (30.5 per

TABLE 1A

Coefficients of correlation for each pair of characters in table 1

	Gelatin	Motility	Indol	Dulcitol	Glycerol	Dextrin	Inulin	Starch
Gelatin		+.99	−.67	−.74	−.98	−.93	−.79	−.98
Motility	+.99		−.66	−.69	−.99	−1.00	−.83	−1.00
Indol	−67	−.66		+.63	+.80	+.71	+.33	+.71
Dulcitol	−.74	−.69	+.63		+.86	+.65	+.62	+.73
Glycerol	−.98	−.99	+.80	+.86		+.97	+.90	+.99
Dextrin	−.93	−1.00	+.71	+.65	+.97		+1.00	+1.00
Inulin	−.79	−.83	+.33	+.62	+.90	+1.00		+.96
Starch	−.98	−1.00	+.71	+.73	+.99	+1.00	+.96	

cent) formed indol from Witte's peptone, and gas was formed as follows: sucrose 148 (98.2 per cent); raffinose 145 (96.2 per cent); dulcitol 45 (29.8 per cent); glycerol 69 (45.6 per cent); salicin 149 (98.8 per cent); dextrin 90 (59.5 per cent); inulin 22 (14.6 per cent) and starch 65 (43 per cent). It is evident from table 1 that sucrose, raffinose and salicin, because of their extreme availability, cannot be employed for differentiation within the group. The coefficients of correlation for each pair of remaining characters are given in table 1A.

Mere inspection of table 1A shows that gelatin liquefaction is almost perfectly correlated with motility and fermentation of glycerol, dextrin, and starch; the association being positive with motility and negative with the others. Similarly motility is

correlated with glycerol, dextrin, starch and gelatin. Each of these characters is correlated with each other. Under these circumstances any of these reactions may be selected for subdivision; the choice depending upon which were employed in an investigation and to some extent on the personal preference of the investigator. The characterization of *B. aerogenes* by Durham as a starch fermenter; the differentiation of *B. aerogenes* from *B. cloacae* by MacConkey on gelatin liquefaction and motility, and by Kligler on glycerol fermentation are all correct; the apparent confusion being the inevitable result of separation upon single characters.

Two species are evidently present, the *B. aerogenes* which rarely, if ever, liquefies gelatin; is non-motile; and forms gas from glycerol and starch; and the *B. cloacae* which liquefies gelatin (often very slowly); is motile; and does not form gas from glycerol nor starch. As gelatin liquefaction is an inconvenient character the organisms are subdivided for further study upon motility into the non-motile *B. aerogenes* and the motile *B. cloacae*. Glycerol or starch would do just as well. Whichever character is selected, a few strains are present in each of the resulting groups which possess some of the salient characteristics of the other. Thus of 89 motile strains 8 did not liquefy gelatin, 8 formed gas from glycerol and 4 from starch, while of 62 non-motile strains, 2 liquefied gelatin, and glycerol and starch were attacked by one.

The presence of a few supposedly non-liquefiers among the motile strains may as probably—and even more probably—be an indication of the inaccuracy and unreliability of the gelatin liquefaction test than of the presence of true intermediate organisms, for the number of gelatin liquefiers recognized increases with the period of incubation. Again is it not reasonable to explain the presence of several glycerol and starch fermenters among the motile strains as due to mixed cultures? Picking off a colony from a plate, even after several replatings, is no absolute criterion that a pure culture was obtained. Some species stick tenaciously together.

One of the motile starch fermenting strains referred to above

was plated out on brilliant green agar. Ten colonies were fished into motility agar and starch; three were non-motile starch fermenters, three were motile and did not attack starch, while four were both motile and starch fermenters thus indicating that, in this instance at least, the presumably overlapping or intermediate

TABLE 2

Showing correlation of indol, dulcitol, and inulin for 62 strains of B. aerogenes

		Indol		Dulcitol		Inulin *	
		+	−	+	−	+	−
Indol	+	31		20	11	7	18
	−		31	14	17	11	18
Dulcitol	+	20	14	34		8	20
	−	11	17		28	10	16
Inulin*	+	7	11	8	10	18	
	−	18	18	20	16		36

* Eight cultures not tested in inulin.

TABLE 2A

Coefficient of correlation for each pair of characters in table 2

	Indol	Dulcitol	Inulin
Indol		+.39	-.22
Dulcitol	+.39		-.22
Inulin	-.22	-.22	

strains are in all probability merely mixed cultures. It is not contended that intermediate strains do not occur; they undoubtedly do; but it is desired to point out that these have been over emphasized in the past and that the plating method cannot always be relied upon to yield pure cultures.

B. cloacae may be defined as a gram negative short rod which ferments lactose weakly; forms acetylmethylcarbinol from glucose; is alkaline to methyl-red; motile; rarely forms indol; prac-

tically always forms gas from sucrose, raffinose, mannitol and salicin; and occasionally from dextrin; gelatin is typically liquefied; and glycerol, inulin and starch are not fermented. As noted above, the few glycerol, inulin and starch fermenters are probably due to mixed cultures and may be dismissed.

The three sucrose negative cultures (also raffinose negative) may be regarded as a variety corresponding to the *B. levans* which MacConkey records as very rare.

The dextrin fermenters probably also constitute a variety of *B. cloacae* but as the composition of dextrin is so variable we hesitate to employ it for differential purposes for the present.

B. aerogenes resembles *B. cloacae* in several respects. It forms acetylmethylcarbinol from glucose; is alkaline to methyl-red; and ferments sucrose, raffinose, mannitol, and salicin with gas formation. On the other hand lactose is more vigorously attacked; gelatin is typically not liquefied; the organisms are non-motile; while glycerol and starch are fermented with gas formation.

Indol was formed by 31 (50 per cent); gas from dulcitol was formed by 31 (50 per cent); and from inulin by 18 (33⅓ per cent) of the *B. aerogenes* strains (8 cultures were not tested with inulin). From tables 2 and 2A which show the distribution with respect to indol, inulin and dulcitol, and the correlation coefficients for these reactions, it is evident that the characters are not associated. They may be of significance for separation of varieties. The utter lack of correlation necessitates the employment of all of these characters, which would lead to the formation of eight varieties. It is deemed unwise to establish such varieties until more extensive collections are studied.

THE COLI GROUP

In the *B. coli* group are included 182 strains quite evenly distributed between the different animal sources, sewage and soil. The group differs sharply from the *B. aerogenes-B. cloacae* series in that the Voges-Proskauer reaction is negative and the methyl-red reaction positive. Starch is not attacked by any of the strains. It has been shown by the author that the Voges-Pros-

kauer negative strains attack the monosaccharids more vigorously, but the disaccharids, trisaccharid, and glucoside no less vigorously than the Voges-Proskauer positive strains.

In table 3 is shown the correlation between the various reactions. As all strains attacked galactose, lactose, maltose and

TABLE 3

Showing correlation of characters among 182 strains of the coli group

		Motility		Indol		Sucrose		Raffinose		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−
Motility	+	130		114	16	77	53	77	53	80	50	92	37	89	41
	−		52	49	3	16	36	19	33	17	35	33	19	25	27
Indol	+	114	49	163		84	79	86	77	87	76	110	52	109	54
	−	16	3		19	9	10	10	9	10	9	15	4	5	14
Sucrose	+	77	16	84	9	93		89	4	64	29	56	36	63	30
	−	53	36	79	10		89	7	82	33	36	69	20	31	38
Raffinose	+	77	19	86	10	89	7	96		65	31	60	35	66	30
	−	53	33	77	9	4	82		86	32	34	65	21	48	38
Dulcitol	+	80	17	87	10	64	33	65	32	97		63	34	73	22
	−	50	35	76	9	29	36	31	34		85	62	22	39	46
Glycerol	+	92	33	110	13	36	69	60	65	63	62	125		89	36
	−	37	19	52	4	36	20	35	21	34	22		56	25	32
Salicin	+	89	25	109	5	63	51	66	48	75	39	89	25	114	
	−	41	27	54	14	30	38	30	38	22	46	36	32		68

mannitol with gas formation and failed to attack the polysaccharids, dextrin, inulin and starch, or to liquefy gelatin, these substances are not included in the table.

The proportion of positive reactions for all strains of the *B. coli* group is as follows: motility 130 (71.5 per cent); indol 163 (89.6 per cent); sucrose 93 (51.1 per cent); raffinose 96 (52.7 per cent); dulcitol 97 (53.3 per cent); glycerol 125 (68.8 per cent) and salicin 114 (62.7 per cent).

The correlation coefficients for each pair of characters is given in table 3A.

The highest coefficient obtained for motility is 0.53 with both

sucrose and dulcitol. Its correlation with other characters is therefore not very marked.

Indol seems to be correlated with salicin, but the small proportion of indol negative strains (10.4 per cent) makes the association of questionable value, and as the coefficients with other substances are extremely low, indol may be eliminated.

Glycerol correlates somewhat with salicin (coefficient 0.52) but

TABLE 3A

Coefficients of correlation for each pair of characters in table 3

	Motility	Indol	Sucrose	Raffinose	Dulcitol	Glycerol	Salicin
Motility		-.39	+.53	+.43	+.53	+.18	+.40
Indol	-.39		+.08	+.00	+.02	-.28	+.76
Sucrose	+.53	+.08		+.99	+.58	-.38	+.20
Raffinose	+.43	+.00	+.99		+.58	-.29	+.27
Dulcitol	+.53	+.02	+.58	+.58		-.21	+.60
Glycerol	+.18	-.28	-.38	-.29	-.21		+.52
Salicin	+.40	+.76	+.20	+.27	+.60	+.52	

with no other character and is therefore not considered desirable for subdivision at this point.

The choice of a differential character is thus narrowed down to sucrose, raffinose, dulcitol and salicin. Sucrose and raffinose are almost perfectly associated (coefficient of correlation 0.99). Consideration of either therefore suffices for both and as the former is slightly better correlated with other characters, sucrose is selected for further discussion.

A comparison of salicin with dulcitol indicates that the alcohol is to be preferred. Salicin correlates better with glycerol and indol (the latter relation of questionable value), while dulcitol has higher coefficients with motility, sucrose and raffinose. A similar consideration leads to the choice of sucrose over salicin.

Sucrose and dulcitol therefore remain. These are the two characters in regard to which there is considerable difference of opinion among students of the *B. coli* group. MacConkey gives preference to sucrose, and in this selection is supported by many investigators (Howe 1912, Kligler 1915, Rogers 1915, etc.); while Jackson (1911) and more recently Giltner (1916) subdivide first on dulcitol. It is quite interesting, therefore, that on the basis of the correlation coefficients there is really little choice between the two. Both are equally well correlated with motility (coefficient 0.53); partially with each other (coefficient 0.58) and not associated with indol. Dulcitol correlates partially with salicin (coefficient 0.60), while sucrose does not (coefficient 0.20). On the other hand, sucrose is almost perfectly correlated with raffinose (coefficient 0.99), whereas salicin is only partially (coefficient 0.58). Although neither can be regarded as associated with glycerol, the coefficient with sucrose (-0.38) is greater than with dulcitol (-0.21).

If our selection is to be guided entirely by correlation, the choice between dulcitol and sucrose is a toss up. Sucrose was finally selected for the primary division because it is more widely distributed in nature, more available to students for investigational purposes, more widely accepted by bacteriologists, and its fermentation better correlated with the source than is dulcitol decomposition. Differentiation on sucrose yields a sucrose positive group of 93 strains, and a sucrose negative group of 89 strains.

THE SUCROSE NEGATIVE STRAINS OF THE COLI GROUP

Of the 89 strains which did not form gas from sucrose, 33 (37.1 per cent) were positive in dulcitol; 69 (77.6 per cent) positive in glycerol; and 51 (57.3 per cent) gave gas in salicin; 53 (59.6 per cent) were motile; only 10 (11.3 per cent) failed to form indol.

The distribution of the organisms, with regard to motility, dulcitol, glycerol, salicin and indol is given in table 4, and the coefficient of correlation for each pair of reactions is given in table 4A. For these strains motility is not distinctly correlated with

any other character. Dulcitol and glycerol are not correlated with each other, nor with indol and motility, but each has a high coefficient of association with salicin. The coefficient for dulcitol

TABLE 4

Showing correlation of characters among 89 sucrose negative strains of the coli group

		Motility		Indol		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−	+	−
Motility	+	53		45	8	22	31	43	10	33	20
	−		36	34	2	11	25	26	10	18	18
Indol	+	45	34	79		29	50	61	18	51	28
	−	8	2		10	4	6	8	2		10
Dulcitol	+	22	11	29	4	33		27	6	28	5
	−	31	25	50	6		56	42	14	23	33
Glycerol	+	43	26	61	8	27	42	69		48	21
	−	10	10	18	2	6	14		20	3	17
Salicin	+	33	18	31		28	23	48	3	51	
	−	20	18	28	10	5	33	21	17		38

TABLE 4A

Coefficients of correlation for each pair of characters in table 4

	Motility	Indol	Dulcitol	Glycerol	Salicin
Motility		−.50	+.22	+.25	+.25
Indol	−.50		−.07	−.08	+1.00
Dulcitol	+.22	−.07		+.20	+.78
Glycerol	+.25	−.08	+.20		+.86
Salicin	+.25	+1.00	+.78	+.86	

with salicin is 0.78 and for glycerol with salicin is 0.86. Indol is also correlated with salicin; all of the 10 indol negative strains are also salicin negative. Differentiation is therefore made upon salicin which gives a sucrose negative, salicin positive subgroup

of 51 strains, and a sucrose negative, salicin negative subgroup of 38 organisms.

The sucrose-negative, salicin-positive subgroup (B. coli). The distribution of the 51 sucrose negative salicin positive strains on motility, dulcitol, and glycerol is indicated in table 5. 33 (64.9 per cent) are motile; 28 (54.9 per cent) form gas from dulcitol, and 48 (94.3 per cent) from glycerol. The extremely small proportion of glycerol negative strains (5.7 per cent) eliminates

TABLE 5

Showing correlation of characters among 51 sucrose negative—salicin positive strains of the coli group. (B. coli)

		Motility		Dulcitol		Glycerol	
		+	−	+	−	+	−
Motility	+	33		18	15	30	3
	−		18	10	8	18	
Dulcitol	+	18	10	28		25	3
	−	15	8		23	23	
Glycerol	+	30	18	25	23	48	
	−	3		3			3

* Coefficient of correlation for motility and dulcitol = 0.02.

this alcohol from further statistical consideration. From table 5 it is seen that motility and dulcitol are not correlated. Further subdivision on correlated characters is not feasible. This entire group then is regarded as the species *B. coli* and two varieties may be formed on motility—the motile *B. coli-communis* and the non-motile *B. coli-immobilis.*

The sucrose negative, salicin negative subgroup (B. acidi-lactici). Of the 38 organisms which were negative for both sucrose and salicin, 20 (52.7 per cent) were motile, 28 (73.7 per cent) formed indol, 21 (55.3 per cent) were positive with glycerol, while only 5 (13.2 per cent) formed gas from dulcitol as shown in table 6. From table 6A it appears that motility is correlated with dulcitol fermentation and indol, and has a slightly higher coefficient with

glycerol than has dulcitol. Indol has a slightly higher coefficient with dulcitol than motility, but the number of dulcitol positive strains is so small, that the coefficients observed cannot be relied upon. Indol and motility seem to be correlated (coefficient

TABLE 6

Showing correlation of characters among 38 sucrose negative, salicin negative strains of the coli group. (B. acidi-lactici)

		Indol		Motility		Dulcitol		Glycerol	
		+	−	+	−	+	−	+	−
Indol	+	28		12	16	2	26	14	14
	−		10	8	2	3	7	7	3
Motility	+	12	8	20		4	16	13	7
	−	16	2		18	1	17	8	10
Dulcitol	+	2	3	4	1	5		2	3
	−	26	7	16	17		33	19	14
Glycerol	+	14	7	13	8	2	19	21	
	−	14	3	7	10	3	14		17

TABLE 6A

Coefficients of correlation for each pair of characters in table 6

	Indol	Motility	Dulcitol	Glycerol
Indol		+.68	+.69	+.40
Motility	+.68		+.62	+.40
Dulcitol	+.69	+.62		+.34
Glycerol	+.40	+.40	+.34	

0.68), and their coefficients with glycerol are identical (0.40). In a preliminary report subdivision was made upon motility into the motile species *B. Gruenthal*, and non-motile *B. acidi-lactici*. It seems best, until more extensive collections are studied, that

all of the sucrose-negative, salicin-negative strains be included in the species *B. acidi-lactici* in which may be recognized two varieties, the motile *B. acidi-lactici* var. *Gruenthali* and the non-motile *B. acidi-lactici* var. *immobili*.

TABLE 7

Showing correlation of characters among 93 sucrose positive strains in the coli group

		Motility		Indol		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−	+	−
Motility	+	77		69	8	58	19	49	27	56	21
	−		16	15	1	6	10	7	9	7	9
Indol	+	69	15	84		58	26	49	34	58	26
	−	8	1		9	6	3	7	2	5	4
Dulcitol	+	58	6	58	6	64		36	28	47	17
	−	19	10	26	3		29	20	8	16	13
Glycerol	+	49	7	49	7	36	20	56		41	15
	−	27	9	34	2	28	8		36	21	15
Salicin	+	56	7	58	5	47	16	41	21	63	
	−	21	9	26	4	17	13	15	15		30

TABLE 7A

Coefficients of correlation for each pair of characters in table 7

	Motility	Indol	Dulcitol	Glycerol	Salicin
Motility		−.27	+.67	+.40	+.54
Indol	−.27		+.05	−.42	+.28
Dulcitol	+.67	+.05		−.32	+.39
Glycerol	+.40	−.42	−.32		+.32
Salicin	+.54	+.28	+.39	+.32	

THE SUCROSE POSITIVE STRAINS OF THE COLI GROUP

Of the 93 organisms which fermented sucrose with gas formation, 77 (82.8 per cent) were motile; 84 (90.4 per cent) formed indol; and positive gas reactions were obtained as follows: dulcitol 64 (72.1 per cent); glycerol 56 (60.2 per cent) and salicin

63 (67.8 per cent). The distribution with respect to these characters and the correlation coefficients are shown in tables 7 and 7A respectively, where it is seen that motility correlates better with dulcitol than does any other of the characters. It also correlates best with salicin. Motility is the best correlated

TABLE 8

Showing correlation of characters among 77 sucrose positive motile strains of the coli group. (B. communior)

		Indol		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−
Indol	+	69		53	16	43	25	51	18
	−		8	5	3	6	2	5	3
Dulcitol	+	53	5	58		32	26	41	17
	−	16	3		19	17	1	15	4
Glycerol	+	43	6	32	17	49		37	13
	−	25	2	26	1		27	19	8
Salicin	+	51	5	41	15	37	19	56	
	−	18	3	17	4	13	8		21

TABLE 8A

Coefficients of correlation for each pair of characters in table 8

	Indol	Dulcitol	Glycerol	Salicin
Indol		+.33	−.27	+.26
Dulcitol	+.33		−.87	−.22
Glycerol	−.27	−.87		+.09
Salicin	+.26	−.22	+.09	

character. There are thus two subgroups, a sucrose-positive, motile subgroup of 77 strains and a sucrose positive non-motile subgroup comprising 16 strains.

The sucrose positive motile subgroup (B. communior). Inspection of tables 8 and 8A shows that among the sucrose positive motile strains, neither indol production nor salicin fermentation is correlated with other characters. Gas formation from dulcitol

and glycerol shows a strong negative association. Those strains which failed to attack glycerol practically always fermented dulcitol. Thus 26 of 27 glycerol negative are dulcitol positive while 17 of 18 dulcitol non-fermenters, tested, formed gas from glycerol. To put it another way, inability to attack either of the alcohols is accompanied by fermentation of the other. Fermentation of either, however, yields but little information as to

TABLE 9

Showing correlation of characters among 16 sucrose positive, non-motile strains, of the coli group

		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−
Dulcitol	+	6		4	2	6	
	−		10	3	7	1	9
Glycerol	+	4	3	7		5	2
	−	2	7		9	2	7
Salicin	+	6	1	5	2	7	
	−		9	2	7		9

TABLE 9A

Coefficients of correlation for each pair of characters in table 9

	Dulcitol	Glycerol	Salicin
Dulcitol		+.65	+1.00
Glycerol	+.65		+.80
Salicin	+1.00	+.80	

how the other will react. The desirability of subdividing on either glycerol or dulcitol to form two species is questioned. For the present the entire group of sucrose fermenting motile forms is designated as *B. communior* and two varieties may be formed on glycerol or dulcitol.

The sucrose-positive non-motile subgroup. Only 16 of the sucrose fermenters were non-motile, and only one of these failed to produce indol. Although the number of organisms is small, it is quite surprising that they should be so evenly divided with respect to gas formation from the test substances. Thus 6

(37.5 per cent) are positive with dulcitol, 7 (43.7 per cent) with glycerol, and 7 (43.7 per cent) with salicin. From tables 9 and 9A it is seen that salicin is the best correlated character. Of the seven salicin positive strains, 6 (85.8 per cent) attack dulcitol and 5 (71.5 per cent) glycerol. The characteristics of this group

TABLE 10

Distribution of organisms from different sources among the various species and varieties

		B. cloacae	B. aerogenes	B. communior	B. neapolitanus	B. coscoroba	B. coli communis	B. coli immobilis	B. acidi-lactici Grunthal	B. acidi-lactici immobili	Total
Soil	No	88	54	26	0	0	2	0	7	0	177
	%	49.7	30.5	14.7			1.1		4.0		
Horse	No	0	0	15	0	0	4	0	0	0	19
	%			79.0			21.0				
Sheep	No	0	0	16	0	5	1	0	0	0	22
	%			72.8		22.7	4.5				
Cow	No	0	0	6	4	0	9	0	1	0	20
	%			30.0	20.0		45.0		5.0		
Pig	No	0	0	9	0	1	11	1	9	0	31
	%			29.0		3.2	35.6	3.2	29.0		
Sewage	No	1	8	3	3	2	1	12	2	7	39
	%	2.6	20.5	7.7	7.7	5.1	2.6	30.8	5.1	17.9	
Man	No	0	0	2	0	1	5	5	1	11	25
	%			8.0		4.0	20.0	20.0	4.0	44.0	
Total		89	62	77	7	9	33	18	20	18	333

therefore resemble the *B. neapolitanus* of MacConkey's varieties. On the other hand, 7 (77.8 per cent) of the 9 salicin negative strains are negative for glycerol while all failed to ferment dulcitol. These are therefore the *B. coscoroba* of MacConkey's classification.

RELATION OF SPECIES TO SOURCE

Table 10 shows the distribution of the organisms from different sources among the various species and varieties. Species or varieties and habitat seem to be somewhat related.

B. aerogenes and *B. cloacae* were obtained only from soil and sewage and were not isolated from any of the animals tested. *B. cloacae* constituted 49.7 per cent of the soil and 2.6 per cent

of the sewage strains, while 30.5 per cent from soil and 20.5 per cent from sewage were *B. aerogenes*.

B. communior was isolated from all sources as follows: soil 11.7 per cent; horse 79 per cent; sheep 72.8 per cent; cow 30 per cent; pig 29 per cent; sewage 7.7 per cent and man 8 per cent. The relative abundance of *B. communior* among the lower animals and scarcity in man and sewage, may well be investigated further.

B. neapolitanus was present only in bovine feces and sewage, comprising 20 per cent of the bovine and 7.7 per cent of the sewage strains.

Of the 9 *B. coscoroba*, 5 were from sheep, 1 from pig, 2 from sewage, and 1 from man. 22.7 per cent of sheep, 3.2 per cent of pig, 5.1 per cent of sewage and 4 per cent of human strains fall in this species.

B. coli, like *B. communior* was isolated from all of the sources tested, but a rather distinct correlation with the source is observed with the varieties *B. coli-communis* and *B. coli-immobilis*. The former comprise 1.1 per cent of soil; 21 per cent of horse; 4.5 per cent of sheep; 45 per cent of cow; 35.6 per cent of pig; 2.6 per cent of sewage; and 20 per cent of human strains. *B. coli-immobilis* was not obtained from the soil, horse, sheep or cow, but it made up 3.2 per cent of the pig, 30.8 per cent of the sewage, and 20 per cent of the human strains.

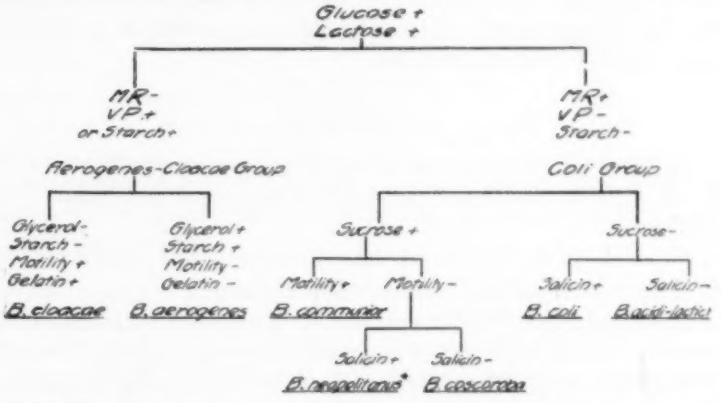
B. acidi-lactici was not obtained from the horse nor sheep, and only rarely from the cow (5. per cent) or soil (4 per cent). The motile variety *B. acidi-lactici var. Gruenthali* was particularly abundant among the pig cultures (29 per cent) and rare in sewage (5.1 per cent) and man (4 per cent). The non-motile *B. acidi-lactici var. immobili* was restricted to man and sewage entirely, comprising 44 per cent of the human and 17.9 per cent of the sewage strains.

If subsequent and more extensive studies confirm these results the determination of species and varieties would have some bearing on the interpretation of the colon test.

The author takes this opportunity to express his gratitude to Dr. R. E. Buchanan for many helpful suggestions and encouragement, and to Prof. G. W. Snedecor for assistance and elucidation of the mathematical principles involved.

SUMMARY

From a statistical study of 333 coli-like bacteria isolated from soil, sewage, and the feces of various animals, the following classification is suggested:



* Designation as species questionable. Probably preferable to regard it as a variety of *B. communior*.

TABLE II

Per cent of positive reactions

	V.P.	Indol	Gelatin	Motility	Starch	Inulin	Dextrin	Salicin	Raffinose	Sucrose	Dulcitol	Glycerol	No. of Strains
B. cloacae	100.0	16.8	91.0	100.0	4.5	4.5	30.4	98.0	93.3	96.7	15.7	9.0	89
B. aerogenes	100.0*	50.0	3.2	0.0	98.5	29.1	100.0	100.0	100.0	100.0	50.0	98.5	62
B. communior	0.0	89.6	0.0	100.0	0.0	0.0	0.0	72.8	94.8	100.0	75.4	63.7	77
B. neapolitanus	0.0	100.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	100.0	85.8	71.5	7
B. cascadae	0.0	89.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0	0.0	22.2	9
B. coli	0.0	100.0	0.0	64.7	0.0	0.0	0.0	100.0	11.8	0.0	55.2	94.3	51
B. acidi lactici	0.0	64.0	0.0	52.7	0.0	0.0	0.0	0.0	2.6	0.0	13.2	55.2	38

* Ten questionable reactions included.

The per cent of positive reactions of the different species is indicated in table 11. *B. neapolitanus* differs from *B. communior*

only with respect to motility, and it may therefore be well to regard it as a variety of *B. communior*. However *B. coscoroba* is so distinctly different from the other sucrose fermenters that its designation as a species seems justified. It should be borne in mind however, that the differentiation in this instance is based on only 9 individual cultures so that the correlations observed must not be over emphasized.

CONCLUSIONS

To treat all characters as of equal taxonomic significance leads to an infinite number of unstable varieties; a condition to be avoided.

Subdivision on correlated characters results in a small number of groups which possess considerable stability.

The species described are quite strikingly correlated with the source, and, if more extensive investigations confirm these observations, recognition of the various species may be of sanitary significance.

It is not supposed that the classification presented is the last word in the differentiation of coli-like bacteria, but it is hoped that if subdivision is to be made upon correlated characters—and there is much to commend such a procedure—the method described in this paper for the determination of the best correlated character, by a study of the coefficients of correlation, will be an aid to later investigators.

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STUDIES ON FOWL CHOLERA

V. THE TOXINS OF BACILLUS AVISEPTICUS[1]

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In presenting the results of his very suggestive studies on the agglutination of bacteria in vivo, Dr. Bull (1916) makes the following statement:

> From table 1 it is seen that bouillon cultures of *Bacillus avisepticus* are highly toxic for rabbits, 0.5 cc. of culture per kilo causing acute death. The intoxication is largely due to a toxin, since 1 cc. per kilo of body weight of a bacteria-free filtrate from a twenty-four hour culture causes acute death.

and again:

> These results especially emphasize the significance of agglutinins and opsonins in the mechanism of natural resistance to infection, since *Bacillus avisepticus* produces a powerful toxin and is still incapable of causing a septicemia of any consequence in the presence of these antibodies.

In view of the difficulty of reconciling these conclusions with studies conducted by other investigators upon *Bacillus avisepticus*, as well as with observations made by the present writer (Hadley 1912, 1914a, 1914b, 1917) in studies on infection and resistance in fowl cholera, it seems desirable to bring into relief the somewhat extraordinary nature of the statements made by Dr. Bull with reference to toxin-production by virulent cultures of the fowl cholera bacteria. This is perhaps especially desirable at this time in view of the many current misconceptions regarding

[1] Contribution 233 from the Agricultural Experiment Station of the Rhode Island State College.

the nature and biological features of the bacterial species in question

In a series of classic papers which appear to have attracted less attention than they deserve among American students of immunological problems, Edmund Weil (1905a, 1905b, 1907a, 1907b, 1908) following the steps of Bail, has presented the clearest evidence as to the elements favoring infection which characterize the activity of the bacteria of the fowl cholera group. An analysis of the situation as based upon his studies and those of his collaborators (Weil and Braun, 1909) may briefly be summarized as follows:

1. Rabbits, inoculated in the pleural cavity with virulent fowl cholera cultures produce an exudate which harbors substances favoring infection—the *aggressins*.

2. These aggressins, which are manifestly equivalent to the virulins of Rosenow and to the antiphagines of Tchistovitch and Yourévitch, possess in high degree the property of furthering infection. Treated animals die with a sub-lethal dose of virus while control animals resist ten times that dose.

3. The infection-furthering property of the exudate does not lie in poisonous substances, since animals easily tolerate 12 cc. or eight times the dose necessary to determine a fatal issue when administered together with a non-lethal dose of virus.

4. The results cannot be due to an inactivating influence in the opsonic sense, since leukocytes (whether in immune or normal animals) manifest no, or slight, phagocytosis either in vitro or in vivo.

5. Toxicity and aggressivity are not identical; toxicity is most noteworthy in organisms which are not highly aggressive; and the most aggressive organisms are non-toxic. *B. avisepticus*, the causative agent in cholera of fowls, is the most aggressive organism that has been studied.

Weil's tests, devised to show whether toxins can be held responsible for the highly fatal results following inoculation with fowl cholera virus, are elaborately planned and (so far as one may judge) carefully executed. Pasteur's observation on the toxicity of the concentrated filtrates of *B. avisepticus* has never been

confirmed under conditions in which it was clearly shown that no bacteria were present in the filtrate. Stang, moreover, was able to inject 20 cc. of concentrated filtrate without producing an infection, or signs of poisoning.

Weil concludes that one cannot demonstrate the slightest toxicity against the most susceptible animals (like the rabbit) in the case of fowl cholera. He pertinently inquires why a toxin-neutralization if present in immune animals should diminish the unlimited development of the bacteria. *B. avisepticus* shows characters sharply opposed to those of toxin-forming bacteria which never develop in great numbers in the body (diphtheria, tetanus, dysentery).

Another line of investigation which demonstrates the lack of extracellular toxins in the case of *B. avisepticus* is found in the experiment of Citron and Pütz (1907) who employed serum extracts and water-extracts of virulent bacterial cultures (artificial aggressins as opposed to natural aggressins of Weil). With these artificial aggressins the authors mentioned inoculated rabbits with the aim of furthering infection, or of producing immunity. The method did not in either case prove so successful as the method of Weil, involving pleural exudates, but the point for our present consideration is that the experiments conducted were such as to demonstrate the total lack of either exotoxins or endotoxins in the materials used for injections. The inoculated animals invariably remained in good health up to the time of infection with the active virus.

Other evidence showing the absence of toxins in the case of *B. avisepticus* is supplied indirectly by the studies of Weil and Braun (1909). Weil (1905b) had already shown that his method of inoculation with sterile pleural exudates was not only sufficient to protect animals against infection, but that the serum of such animals possessed protective power. The question naturally arose regarding the nature of the protective substances in such serum. Weil and Braun attempted to ascertain whether the protection was due to bactericidal substances. Animals were accordingly inoculated with serum which had been "treated" (absorbed) with fowl cholera bacteria, and survived infection.

When the treated serum was tested for its bactericidal properties it was found that they had quite disappeared. Untreated serum still showed a slight growth-inhibiting power. The authors were therefore led to conclude that bactericidal antibodies cannot be considered in the protective action since they can be separated from such serum without causing the degree of protection to be diminished. In a similar manner Weil was able to show later that old immune serum might retain its bactericidal properties after it had lost all protective power.

The same authors demonstrated by detailed experiments that there is no opsonic action in fowl cholera immune serum. It was clearly shown that there was no phagocytosis of the fowl cholera bacteria in either normal or immunized animals. Sulima (1909) on the contrary, showed that some phagocytosis took place in immune animals but that it was not marked. It is obvious that in dealing with such small organisms as the bacteria of fowl cholera (0.3 to 0.5 μ) one must use suitable methods of staining, and much care in examining the leukocytes for evidences of phagocytosis.

In the work of Huntemüller (1906) also there is good evidence of the lack of toxin-production by *B. avisepticus*. One phase of this writer's investigations involved the injection into rabbits of large amounts of bacterial filtrates in order to ascertain whether protective substances could be washed out of the cells. This was not found to be the case; but the point of present interest lies in the fact that the injection of the filtrates produced no ill effect as they certainly would have done if the organisms used had been capable of elaborating so powerful an exotoxin as Dr. Bull's statements imply.

Taken as a whole, the results of studies on infection and immunity in fowl cholera, although appearing to demonstrate that the protective function of fowl cholera immune serum does not rest upon the action of opsonins, nor upon bacteriolytic or bactericidal components (thus, by elimination, suggesting an antitoxic immunity), have failed to reveal the activity of any agent that can properly be regarded as a toxin, either intra- or extra-cellular.

Within the past few years the present writer (Hadley and

Amison, 1911, Hadley, 1912, 1914a, 1914b) has devoted some attention to the problems of infection and resistance in the organisms of the hemorrhagic septicemia group. Use has been made of a highly virulent culture (strain 48) of *B. avisepticus*. The lethal dose of this culture for rabbits has frequently stood at 0.000,000,000,001 cc. of a forty-eight-hour broth culture. Gallagher (1917) in recent experiments, in which he employed the same culture as the infective agent to test the immunizing power of the writer's Culture 52, as well as of certain strains of cattle, sheep and swine septicemia, used the strain in amounts of 0.000,000,001 cc. with regularly successful infections. Such a strain should be well adapted for testing out the question of toxin-production by *B. avisepticus*.

EXPERIMENTAL

In studies involving the attempt to immunize rabbits and pigeons with killed cultures of strain 48, not yet reported, the writer has had occasion to administer large doses of killed broth culture. The following is a protocol:

Eight rabbits (923–931) each received subcutaneously on October 17, 1912, 1 cc. of a forty-eight hour broth culture of strain 48 killed by heating at 63°C. for thirty minutes; no illness resulted. Dose repeated on November 21 and again on December 5; no result.

On December 20, 1912, all the animals, together with two controls, were infected by inoculation with 0.01 cc. of forty-eight hour broth culture of strain 48. The controls and all but two of the principals died in fourteen to forty hours.

From the above test it may be concluded that the cultures killed at a temperature usually regarded as too low to destroy in toto extracellular toxins, if present, showed no toxicity for rabbits in the amounts used; furthermore that they failed in all but two instances to afford protection against infection with living culture.

Tests were also performed on pigeons, which are highly susceptible to intramuscular inoculation with the fowl cholera virus.

Six pigeons were inoculated subcutaneously, at intervals of

seven to ten days with forty-eight-hour broth cultures of strain 48, killed by heating at 63° for one-half hour.[2]

PROTECTIVE INOCULATIONS							INFECTION WITH CULTURE 48. MARCH 17		
INOCULATION RECORD	Dates and amounts					Method	Method	Result	
	2-3	2-10	2-17	2-24	3-3			Date of Death	Period
1240	1.0	2.0	2.0	2.0	2.0	S	S	March 23	Six days
1241	1.0	2.0	2.0	2.0	2.0	M	S	March 28	Eleven days
1242	1.0	2.0	2.0	2.0		S	S	March 18	One day
1243*	1.0	2.0	2.0	2.0		M	S	April 4*	Survived
1244	1.0	2.0				S	S	March 18	One day
1245	1.0	2.0				M	S	March 23	Six days
1276							S	March 18	One day
1277							M	March 18	One day

S, subcutaneous (in the neck).
M, intramuscular (in the breast muscle).
* Reinforced with 0.000,01 cc. culture 48, March 30.

No reactions followed the immunizing inoculations with killed cultures. Two weeks after the last immunizing dose all the principals together with two controls were infected with 0.000,01 cc. of culture 48, as shown in the table, and with the results indicated.[3]

These protocols are similar to many others on record and indicate how free the *B. avisepticus* cultures were from anything that could be regarded as bacterial toxins. One can readily imagine the results that would follow had *B. diphtheriae* or some other toxic culture been used in these experiments in place of the fowl cholera bacterium

The instances mentioned above involved the use of killed cultures and the doses were not administered intravenously as was the case in Dr. Bull's tests on rabbits and dogs in which he obtained results that suggested acute intoxication. In order

[2] In this particular test, in order to obtain a more concentrated suspension of bacteria, the cultures were centrifuged and two-thirds of the supernatant medium poured off. The sediment was re-suspended in the remaining one-third and injected.

[3] These data will be presented at a later date in connection with the subject of immunization by means of killed cultures.

to reproduce more faithfully the experimental conditions under which he worked the writer inoculated intravenously two rabbits with 1 cc. per kilo body weight of a forty-eight-hour killed broth culture of the highly virulent strain 48 killed by heating at 62° for 20 minutes. The results are indicated in the accompanying table.

INOCULATION RECORD	WEIGHT	INOCULATION (INTRAVENOUS)			HISTORY OF ANIMAL
		Time	Culture	Amount	
	grams			*cc.*	
1584A	950	May 1, 4 p.m.	Killed at 62°C. for 20 minutes	1.0	5.00 p.m., sick 11.00 p.m., no change. 7.00 a.m., May 2, quiet, does not eat 7.00 a.m., May 3, condition improved
1585A	880	May 1, 4 p.m.	Living	0.9	5.00 p.m., sick 11.00 p.m., very weak, breathing with difficulty 7.00 a.m., dead; general septicemia

From the data presented in the tabulation it is apparent that the intravenous injection of killed culture produced no indications of strong toxicity; moreover, that inoculation with a relatively large amount of living culture produced fatal results only after a period of fourteen hours, indicating no strong toxic action in the unmodified virus.

In addition to the tests mentioned above, another experiment may be cited which demonstrates even more conclusively the absence of a strong extra-cellular toxin derived from a highly virulent culture of *B. avisepticus* growing in broth culture.

A rabbit was inoculated by the intra-abdominal route with 0.001 cc. of a broth culture of strain 48 and died in fourteen hours. The culture was regained from the heart's blood and plated. A colony was subcultured into flasks of chicken broth. One of these was grown at 37°C. for forty-eight hours and passed through a Berkefeld N-candle under suction. The filtrate was tested for sterility as follows: Ten 0.1 cc. samples of filtrate were trans-

ferred to tubes of chicken broth and incubated at 37°C. for ninety-six hours. No growth appeared at the end of that time. In the meantime the remaining filtrate was divided into three portions: portion a was placed in the ice-box; portion b was incubated for twenty-four hours at 37°C., and portion c was heated at 62°C. for twenty minutes. The incubated filtrate showed no growth. It was intended to inject all three portions into rabbits, but, owing to an accident, only the ice-box filtrate and the heated filtrate were employed for rabbits. These were injected intravenously in amounts of 1 cc. as shown in the accompanying table. Of the incubator filtrate 1 cc. was injected intravenously into an adult hen:

INOCULATION RECORD	WEIGHT	FILTRATE	DATE	HISTORY OF ANIMAL
	grams			
1580	2100	Heated	April 5, 4 p.m.	Remains normal
1581	1390	Ice-box	April 5	Remains normal
411H		Incubator	April 5	Remains normal

From these results there is no evidence of a toxic filtrate from the growth of *B. avisepticus*.

The tests described above were made with filtrate from a forty-eight-hour broth culture. Assuming that sufficient time might not have been given for toxin-formation, the second flask was incubated for four days at 37°C., and the culture passed through a Berkefeld N-candle under suction. The fifteen-minute filtrate was tested for sterility by the inoculation of five tubes of chicken broth with 0.1 cc. samples. No growth resulted after forty-eight hours' incubation. The remaining filtrate was divided into two lots. One was placed in the incubator and the other in the ice-box for about twenty-four hours, until used. Intravenous inoculations were made as shown in the following tabulation:

INOCULATION RECORD	WEIGHT	FILTRATE	AMOUNT	TIME	HISTORY OF ANIMAL
	grams		cc.		
1581A	1340	Incubated	1.5	April 7	
				11.30 a.m.	Inoculated
				1.45 p.m.	Dull, breathing with difficulty; eyes closed
				3.30 p.m.	No change
				6.30 p.m.	More active
				April 8	
				9.00 a.m.	Quiet, but otherwise normal
1581B	1225	Ice-box	2.0	April 7	
				11.30 a.m.	Inoculated
				1.15 p.m.	Dull, breathing with difficulty; unable to stand
				3.30 p.m.	No change
				6.30 p.m.	Upright, but staggers; breathes with difficulty
				April 8	
				9.00 a.m.	Better; quiet
				April 9	
				9.00 a.m.	Appears normal

In the results of this test it appears that a reaction closely simulating an anaphylactic shock resulted from the inoculation of the second filtrate. The result was not fatal, however, and it may be questioned whether it was due to the presence of an exotoxin or to some other factor at present unrecognized.

In view, however, of the discrepancy between the first and second tests, a third experiment was undertaken in which use was made of a still larger amount of filtrate from a six-day chicken broth culture passed through a Berkefeld N-candle. The filtrate was tested for sterility, and divided into two portions, one of which was incubated over night and the other placed in the ice-box. A slight haziness developed in the incubator tubes and subcultures were made in chicken broth. These, however, remained sterile. A 1314-gram rabbit was accordingly inoculated intravenously with 1.5 cc. of the incubator filtrate. No sign of illness followed within the following twenty-four hours during which the animal was under observation.

As a final test, in order to explain, if possible, the discrepancies between Bull's results and those of the present writer, it seemed desirable to repeat the experiment with the same culture used by Bull. Fortunately, as a result of the careful records kept by the laboratory of the Curator of Public Health, at the American Museum of Natural History, it was possible to locate the culture used by Bull, who had in the meantime discarded his strain, and to obtain a subculture, together with a brief history of organism. This seemed especially desirable in view of the many errors that are made in the recognition of the organism of the authentic fowl cholera. It can scarcely be doubted that the majority of the cultures so labelled, that may be obtained from any laboratory, are not *B. avisepticus*.

Upon receipt of the culture it was plated and subcultured in chicken agar. Flasks of chicken broth were inoculated and incubated for forty-eight hours at 37°C. One of the cultures was then passed through a Berkefeld N-candle under suction and this filtrate tested for sterility. The filtrate was then divided into two portions, one of which was inoculated at 37°C. over night as a final test of sterility; the other kept in the ice-box. The following morning, all evidences having pointed toward the sterility of the filtrate, a 1.5 cc. sample was injected intravenously into a 1375-gram rabbit, which died in less than three hours with symptoms of intoxication.

Another rabbit of 1347 grams was inoculated intravenously with 0.7 cc. of a twenty-four-hour chicken broth culture of Dr. Bull's strain and no ill effects resulted. Apparently 1.5 cc. of a forty-eight-hour culture-filtrate contained a lethal dose of toxin, while 0.7 cc. of a twenty-four-hour culture did not. This is suggestive of an endotoxin rather than an exotoxin. To demonstrate this further, a forty-eight-hour culture of Dr. Bull's strain was shaken for one-half hour, incubated at room temperature for forty-eight hours and shaken again for three hours. It was then passed through a Berkefeld N-candle and the filtrate tested for sterility by subcultures. Of the sterile filtrate 0.5 cc. was then injected into the ear vein of a 1000-gram rabbit. One hour after the

injection the rabbit showed definite symptoms of intoxication and died in about six hours.

The tests reported in the previous pages were sufficient to demonstrate a total lack of toxicity in the culture or filtrates of *B. avisepticus* (strain 48) employed, but to show the presence of a strong toxic substance in the filtrate of forty-eight-hour cultures of the organism used by Dr. Bull. The contrasts may be summarized as follows:

ORGANISM	FILTRATE	CULTURE
B. avisepticus (strain no. 48)	Non-toxic in amounts of 1 cc. per kilo of body-weight	Highly infectious in amounts of (at least) 0.000,000,001 cc. for rabbit of any weight, producing death in fourteen hours
Dr. Bull's culture	Toxic in amounts of 0.5 to 1 cc. per kilo of body weight. Fatal in two to six hours	Small amounts of twenty-four hour culture harmless.*

* May perhaps become lethal after several passages through rabbits.

In view of these discrepancies, it seemed desirable to examine culturally the strain employed by Dr. Bull. It was therefore submitted to the usual cultural and biochemical tests; and in addition was tested for its agglutinative reactions with a number of immune sera, including those of *B. avisepticus* and *Bact. gallinarum* (fowl typhoid). These tests will now be described.

Morphological, cultural and biochemical features of Dr. Bull's strain. Slides prepared from twenty-four-hour chicken agar cultures and stained with gentian violet revealed a short plump rod, having an average size of 0.7 by 0.45μ. a maximum size of 1.2 by 0.5 μ, and a minimum size of 0.4 by 0.3 μ. The ends of the rods were rounded and many of the shorter rods resembled cocci. Pairs of rods were common; chains of three seldom seen; filaments occasionally. Peripheral staining was observed.

The culture grew well on chicken agar; resembled in all respects growth of *Bact. pullorum* or *B. typhosus*. In broth there was a fair clouding at the end of twenty-four hours.

No gas was formed in glucose, sucrose or lactose (Smith tubes). Titration of culture from the open arm of these cultures, and of cultures in other sugar broths (in plain tubes) at the end of five days gave the following results:

CARBOHYDRATE USED IN THE TEST	DR. BULL'S CULTURE	*B. avisepticus* (FOWL CHOLERA)	*Bact. gallinarum* (FOWL TYPHOID)
Glucose	0.8	0.3	1.6
Lactose	0.0		0.0
Sucrose	0.1	0.8	0.0
Dulcitol	0.5	0.9, 0.4, 0.4	0.9, 1.9, 0.9
Dextrin	0.9	0.0, 0.2, 0.2	0.8, 0.8, 0.7
Maltose	1.1	0.1, 0.1, 0.1	0.9, 0.8, 0.8

Litmus milk inoculated with one loopful of a twenty-four-hour broth culture showed a slight acidity in twelve hours at 37°C. This increased slightly for the next twelve to twenty-four hours but after thirty-six to forty-eight hours there was a tendency to return to neutral. The cream ring, however, began to turn blue[4] about twenty-four hours after the inoculation of the tubes and the color steadily increased so to as give a blueness of grade 2[5] before the body of the milk had returned to neutral. Neutralization occurred on the sixth day after inoculation. On the seventh the body of the milk as well as the cream ring was slightly alkaline and on the eighth distinctly alkaline (grade 1). Grade 3 alkaline was attained on the thirtieth day.

Without entering into further details regarding the biochemical reactions, a sufficient number of data have been presented to indicate clearly that the culture with which Dr. Bull worked was not *B. avisepticus* in the sense that this is the causative agent in

[4] It is interesting to note that in alkalining cultures such as *B. pullorum*, *B. gallinarum* and the paratyphoids A and B, the alkalinity may be shown in the cream ring for some days before the color of the body of the milk has even returned to neutral, and many days before the body of the milk becomes alkaline.

[5] See Hadley (1917).

fowl cholera and a member of the hemorrhagic septicemia group. *B. avisepticus* does not ferment most of the carbohydrates ordinarily used in bacteriological work; and it leaves litmus milk quite unchanged after a period of ninety days at 37°C.

Agglutination tests. In view of the fact that fowl cholera immune serum seldom if ever agglutinates completely (in vitro) its homologous antigen in dilutions greater than 1 : 160, the specific agglutination test is obviously of slight value in testing a questionable strain. It is necessary to demonstrate an affinity or lack of affinity with other immune sera. Since the majority of so-called fowl cholera cultures maintained in the laboratories of this country are not *B. avisepticus* but *Bact. gallinarum* (E. Klein), or some other member of the colon-typhoid intermediates, Dr. Bull's culture was first tested against sera immune to the bacillus of fowl typhoid. Since the laboratory collection of immunized animals contained rabbits immune to two fowl typhoid strains obtained from Dr. Theobald Smith, these, among others, were employed for the tests with results as shown in the following table.

From a study of the agglutination features as presented in the table it appears that there was present a satisfactory agglutination affinity between all of the sera immune to the fowl typhoid strains (*Bact. gallinarum*) (115, 116, 102A[6] and 118[6]) and Dr. Bull's culture, but none with *Bact. avisepticus* antigen. Moreover, that serum immune to *B. avisepticus* while agglutinating its homologous antigen at $\frac{C}{40}$, $\frac{4}{80}$, $\frac{2}{160}$, failed to agglutinate Dr. Bull's culture in any significant dilution.

SERUM AND ANTIGEN		SERUM—DILUTIONS								DATE
Culture Number	Designation	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	Control	
115	Fowl typhoid, vs.									
	Homolog. antigen.....	C	C	C	C	C	C	4	T	12-22-16
	B. avisept. antigen.....	0	0	0	0	0	0	–	0	1-19-17
	Bull. cult. antigen.....	C	C	C	C	C	–	–	0	3-30-17
	Hog cholera antigen.....	T	0	0	0	0	–	–	0	2-16-17
116	Fowl typhoid, vs.									
	Homolog. antigen.....	C	C	C	4	2	T	–	T	4-20-17
	B. avisept. antigen.....	0	0	0	0	0	0	–	0	1-20-17
	Bull. cult. antigen.....	C	C	4	2	1	–	–	0	4-20-17
	Human typhoid antigen.	1	T	0	0	0	0	–	0	3- 2-17
102A[6]	Fowl typhoid (?), vs.									
	Homolog. antigen.....	C	C	C	C	4	2	T	0	7-31-16
	B. avisept. antigen.....	2	1	0	0	0	–	–	0	11- 7-15
	Bull. cult. antigen.....	–	C	C	C	C	–	–	0	3-12-17
	Human typhoid antigen.	0	0	0	0	0	–	–	0	3-12-17
118[6]	Fowl typhoid, vs.									
	Homolog. antigen.....	C	C	C	C	C	4	3	0	4-23-17
	B. avisept. antigen.....	0	0	0	0	0	–	–	0	4-23-17
	Bull. cult. antigen.....	C	C	C	C	C	–	–	0	4-23-17
	Bact. pullorum antigen...	C	C	C	C	C	–	–	0	4-23-17
Mxd.	*Bact. pullorum*, vs.									
	Homolog. antigen.....	C	C	C	C	C	–	–	0	3- 3-17
	B. avisept. antigen.....	0	0	0	0	0	–	–	0	1-22-17
	Bull. cult. antigen.....	1	T	0	0	0	–	–	0	3-10-17
	Fowl typh. antigen.....	C	C	C	C	4	0	–	0	3- 3-17
48, 52	*B. avisepticus*, vs.									
	Homolog. antigen.....	C	4	2	T	0	0	–	T	4-20-17
	Bact. pull. antigen.....	0	0	0	0	0	0	–	0	3-26-17
	Bull. cult. antigen.....	0	0	0	0	–	–	–	T	4-20-17
	Fowl typh. antigen.....	0	0	0	0	–	–	–	0	3-26-17

C = Complete sedimentation.
0 = No agglutination.
4, 3, 2, 1 = Intermediate grade of agglutination.
– = No test.
T = Trace of sediment (control tube.)

[6] Probably *Bact. pullorum* (Note at time of correcting proof).

CONCLUSIONS

These results of the agglutination tests appear to substantiate the results of the cultural tests in demonstrating that Dr. Bull's culture was a strain of the fowl typhoid bacterium and not a representative of the fowl cholera or hemorrhagic septicemia group. The facts that cultures of the fowl cholera bacterium are non-toxic, and that they are not opsonized into phagocytosis, therefore remain uncontroverted so far as Dr. Bull's experiments are concerned. Indeed Tchistovitch (1909) appears to have demonstrated that, in the case of the actual fowl cholera organism, serum from the dog did not assist the rabbit leukocytes to ingest the fowl cholera organisms. Notwithstanding this circumstance, however, Dr. Bull's study of phagocytosis in reference to the culture used by him, if properly interpreted, adds much to our knowledge of the toxicity, opsonins and phagocytosis of one of the most interesting and slightly studied types of the colon-typhoid intermediates.

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A LACTOSE FERMENTING YEAST PRODUCING FOAMY CREAM

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Large amounts of cream are lost during the hot summer months from an undesirable fermentation known as "foamy cream." This loss is due chiefly to the mechanical action of the fermentation, rather than to the effect of undesirable odors and flavors, for often one-third to one-half of the cream is lost from the can through foaming while in transit. The fermentation is best identified by this characteristic foaming action and by the yeasty or fruity odor imparted to the cream.

EXAMINATION OF CREAM

Numerous samples of foamy cream collected from different parts of Kansas by the State Dairy Commissioner have been sent to the bacteriological laboratory for analysis. The microbial flora as revealed by direct microscopic examination and by plate cultures proved to be similar for all samples. The cream was plated on whey and lactose agar respectively. The latter medium was acidified with 1 cc. of a 1 per cent tartaric acid solution per tube of medium.

Yeast cells were very prominent, as well as rod shaped bacteria, which appeared as short or long filaments and showed numerous granules on staining. The latter organism upon isolation proved to be a member of the *B. bulgaricus* group.

All cream showing such a flora caused foaminess when placed in raw or sterile cream; and the inoculated cream always exhibited a microbial flora identical with that of the original product.

The predominating type proved upon isolation to be a lactose fermenting yeast. Its ability to produce foamy cream was easily demonstrated by placing it in raw or sterile cream. The characteristic action of the organism is noted in figure 1.

FIG. 1

PREVIOUS WORK ON LACTOSE FERMENTING YEASTS

In this country, very few investigators have reported the presence of yeasts of this type. An abnormal fermentation in Swiss cheese, as reported by Russell and Hastings (1905) was due to a lactose fermenting yeast. Harrison (1902) observed yeasts capable of fermenting lactose in large numbers in American cheese and milk. He attributed a bitter fermentation in milk and dairy products to their presence.

The majority of lactose fermenting yeasts have been isolated and studied by European investigators. Grotenfelt (1889) described such a yeast isolated from milk and named it *Saccharomyces acidi-lactici*. Beijerinck (1889) isolated a lactose yeast from kefir grains, and one from Edam cheese. The former is known as *Saccharomyces kephyr* and the latter as *Saccharomyces tyrocola*.

While Beijerinck states that both of these yeasts were Saccharomyces, other investigators who have studied them assert that both are incapable of sporulating and hence should be classed as Torulae. A non-spore producing yeast was isolated from Grana cheese by Bochicchio (1894) and named *Lactomyces inflans-caseigrana*. A typical lactose fermenting Saccharomyces obtained from Emmenthaler cheese, is described by Freudenrich and Jensen (1897). Jensen (1902) also noted two species in butter which were true yeasts. Maze (1903) studied ten different torula from soft cheese. One fermented lactose only, while the others fermented glucose, levulose, maltose and sucrose. Duclaux (1900) describes three lactose fermenting yeasts, isolated by Kayser, Adametz and himself. All are non-spore producing yeasts and capable of fermenting glucose, lactose, sucrose, galactose, invert sugar and maltose, slightly. All are apparently different species however. Another typical saccharomyces, *Saccharomyces fragilis*, was isolated from kefir, by Jorgensen (1911).

According to Lafar (1911) the yeasts of Jorgensen, Freudenreich and Jensen, and Jensen and Maze, are the only lactose fermenting yeasts which can definitely be reported as Saccharomyces.

Yeasts capable of fermenting lactose, according to the present classification are grouped as either true or false yeasts. If true yeasts, they sporulate and hence are considered as belonging to Hansen's fifth subgroup of Saccharomyces, if false yeasts, they are non-sporulating varieties, or Torulae. Of the lactose fermenting yeasts studied, representatives of both genera are known. The differentiating factor is sporulation and the observations of different investigators, upon this point, for the same yeasts do not agree. Hansen states that the torula may be only a tempo-

rary stage of development of yeasts. He has demonstrated that, at the least, spore production is not a stable factor, for he has been able to produce an asporogenic race of Saccharomyces by varying the condition of cultivation. This would lead one to doubt the usefulness of attempting to use sporulation in yeasts as of much diagnostic value.

MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL CHARACTERISTICS

The yeast causing "foamy cream" is oval to elliptical in shape, averaging 5 microns in length and 2 microns in breadth.

Typical spores were not demonstrated by cultivation upon gypsum blocks or potato at temperatures of 25°C. and 35°C. According to the present means of classifying yeasts, the organism is therefore a false yeast or torula. The average colony varies from 2 to 3 mm. in diameter; but more minute forms are frequently noted. In appearance the colonies are spherical with smooth edges, having a raised smooth glistening surface. Plate cultures of the organism emit a yeasty or fruity odor. Upon lactose agar it grows moderately, exhibiting a raised, smooth, and dull to glistening growth. It produces a slight cloudiness in sugar broth; no pellicle, acid reaction in litmus milk; and fails to liquefy gelatin. In milk about 0.3 per cent acid is produced. Gas production was demonstrated in glucose, lactose, sucrose, levulose, galactose, maltose and bile lactose; although the action in galactose, maltose and bile lactose was slow and feeble.

The relation of temperature to the rate of fermentation of the organism is represented in table 1. Litmus milk fermentation tubes were inoculated with 0.1 cc. broth culture of the yeast and incubated at 18°C., 25°C. and 37°C., respectively. The results are self explanatory and easily account for the predominance of foamy cream during hot weather. The highest temperature studied, 37°C., is not necessarily the optimum temperature for the development of the organism, but lack of incubation facilities prevented a more exact study of this point.

TABLE 1

The relation of temperature to fermentation

TEMPERATURE OF INCUBATION	DAYS OF INCUBATION						
	1	2	3	4	5	6	7
deg. C.							
18	No change	No change	No change	No change	No change	No change	No change
25	Slight acid	Acid, gas slight	Acid, gas 50 per cent*	Acid, gas 100 per cent			
37	Acid	Acid, gas 100 per cent					

*The percentage of gas refers to the amount formed in the closed arm of a Smith fermentation tube.

POWERS OF RESISTANCE

Thermal death point

Twenty-four hour lactose broth cultures were thoroughly shaken and equal portions placed in sterile capillary pipettes of uniform capacity and thickness of glass. Both ends were sealed by heat and the sealed tubes were then placed in water baths of constant temperatures for ten minutes. At the end of ten minutes the tubes were removed from the water bath and cooled by placing in cold water. The pipettes were opened aseptically and tubes of litmus milk were inoculated with the contents and incubated at 37° for four days. No growth was observed in cultures made from these tubes after exposure to a temperature of 55°C. or higher. The thermal death point therefore lies between 50° and 55°C.

Resistance toward chemicals

The yeast's resistance toward various chemicals was determined by using a twenty-four-hour lactose broth culture of the organism. This was thoroughly mixed to break up all clumps and masses. Three standardized platinum loops were used for

inoculating the tubes of disinfectants and for transferring the organisms from the disinfectants to the culture media. Sterile litmus milk was used as the culture medium.

The disinfectants employed were: washing soda, calcium hypochlorite, lime, boric acid, cresol and carbolic acid. The vitality of the exposed cultures was determined by incubating

TABLE 2

Resistance towards disinfectants

DISINFECTANT	STRENGTH OF DISINFECTANT	MINUTES EXPOSED TO DISINFECTANTS											
		1	3	5	10	15	30	45	60	75	90	150	120
	per cent												
Washing soda	1	+	+	+	+	+	+	+	+	+	+	+	+
Washing soda	2	+	+	+	+	+	+	+	+	+	+	+	+
Washing soda	5	+	+	+	+	+	+	+	−	−	−	−	
Calcium hypochlorite	1	+	−	−	−	−	−	−	−	−	−	−	−
Calcium hypochlorite	2	−	−	−	−	−	−	−	−	−	−	−	−
Calcium hypochlorite	5	−	−	−	−	−	−	−	−	−	−	−	−
Lime	1	+	−	−	−	−	−	−	−	−	−	−	−
Lime	2	+	−	−	−	−	−	−	−	−	−	−	−
Lime	5	+	−	−	−	−	−	−	−	−	−	−	−
Boric acid	1	+	+	+	+	+	+	+	+	+	+	+	+
Boric acid	2	+	+	+	+	+	+	+	+	+	+	+	+
Boric acid	5	+	+	+	+	+	+	+	+	+	+	+	+
Cresol	0.5	+	+	+	+	+	+	+	+	+	+	+	+
Cresol	1	+	−	−	−	−	−	−	−	−	−	−	−
Cresol	2	−	−	−	−	−	−	−	−	−	−	−	−
Carbolic acid	5	−	−	−	−	−	−	−	−	−	−	−	−

Growth +; no growth −.

the litmus milk tubes for four days at 37° and noting the presence or absence of growth.

The results are noted in table 2.

The results in table 2 indicate that washing soda exhibits little disinfectant action on the yeast. Exposure for two hours to both 1 and 2 per cent solutions failed to kill the organism, while a 5 per cent solution prevented their growth in seventy-five minutes.

Calcium hypochlorite prevents the development of the yeast in 2 and 5 per cent solutions within one minute, while a 1 per cent solution acts similarly in three minutes.

Lime possesses disinfectant properties in 1, 2 and 5 per cent solutions with three minutes' exposure.

Boric acid exhibits no killing powers in strengths of 1, 2 and 5 per cent after two hours.

Cresol in 0.5 per cent solution fails to destroy the yeast in two hours while 1 per cent kills in three minutes and 2 per cent in one minute.

Carbolic acid in 5 per cent solution is likewise efficient within one minute.

Resistance toward desiccation

Data showing the ability of the yeast to withstand desiccation are somewhat limited. However, the results obtained demonstrate it to be very resistant to drying. Soil and alfalfa stems were placed in sterile containers and inoculated with milk cultures of the yeast. The absence of yeasts in the soil and alfalfa used was assured before inoculation. The substances were examined at frequent intervals to note the effect of desiccation. The yeasts were observed after eighty-six days in large numbers in both substances. No further analysis of the materials was made.

CONCLUSIONS

1. A lactose-fermenting yeast is the essential organism in the abnormal fermentation of cream, known as "foamy cream."
2. Raw or sterile cream inoculated with a pure culture of the yeast shows typical foaming characteristics.
3. The optimum temperature for growth is near 37°C. This accounts for the prevalence of foamy cream during hot weather only.
4. The thermal death point of the yeast is near 55°C. for ten minutes.
5. The yeast offers slight resistance toward efficient disinfectants.
6. The organism is quite resistant towards desiccation.

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STUDIES IN THE CLASSIFICATION AND NOMENCLATURE OF THE BACTERIA

VII. THE SUBGROUPS AND GENERA OF THE CHLAMYDOBACTERIALES

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Order II. **Chlamydobacteriales.** *ordo nov.*

Synonyms:

Cladotrichiae Trevisan, 1879, p. 15
Cladotricheen Zopf, 1883, p. 45
Cladothrichacei Schroeter, 1886, p. 173
Cladothricheae Hueppe, 1886, p. 140
Leptotrichacei Schroeter, 1886, p. 170
Crenotricheae DeToni and Trevisan, 1889, p. 925
Chlamydobacteriaceae Migula, 1894, p. 237
Desmobacteriaceae Benecke, 1912, p. 188 in part
Phycobacteriales Engler

Filamentous bacteria, alga-like, typically water forms, frequently sheathed, without true branching although false branching may be present. The sheath is frequently impregnated with iron. Conidia may be developed, but never endospores. Sulphur granules or bacteriopurpurin never present. Mature cells or filaments not motile, not protozoan-like.

The order contains a single family *Chlamydobacteriaceae.*

Family I. **Chlamydobacteriaceae** Migula, 1894, p. 237

The following genera have been included in the family by various authors:

Gaillonella Bory, 1823
Sphaerotilus Kuetzing, 1833, p. 385
Gallionella Ehrenberg, 1838, p. 166
Leptothrix Kuetzing, 1843, p. 198
Didymohelix Griffith, 1853, p. 438
Crenothrix Cohn, 1872, p. 130
Siphonomyxa Billroth, 1874, p. 27
Cladothrix Cohn, 1875, p. 185
Leptotrichia Trevisan, 1879, p. 138, in part
Phragmidiothrix, Engler 1882, p. 19
Kurthia Trevisan, 1885, p. 92
Billetia Trevisan, 1889, p. 11
Detoniella De Toni and Trevisan, 1889, p. 929
Leptotrichella De Toni and Trevisan, 1889, p. 935
Streblothrichia Guignard, 1890, p. 124.
Eucrenothrix Hansgirg, 1891, p. 313
Chlamydothrix Migula, 1900, p. 1030
Clonothrix Schorler, 1904, p. 691.
Spirophyllum Ellis, 1907, p. 516
Nodofolium Ellis, 1910, p. 321.
Leucothrix Oersted, ——, p. 44.

Of these the following will be disregarded because of the inadequate characterization of genus and species: *Billetia*, *Kurthia*, *Siphonomyxa*, *Streblothrichia*.

The following are algal genera to which certain of these organisms were incorrectly assigned: *Gaillonella*, *Gallionella*.

The following names are invalid because of the prior use of the names for distinct groups.

Spirophyllum Ellis, 1907, p. 516
not *Spirophyllum* Schindler, 1905, p. 82
Cladothrix Cohn, 1875, p. 185
not *Cladothrix* Nuttall, 1849

The following are subgenera: *Eucrenothrix*, *Leptotrichiella*, *Leucothrix*.

The following generic names must be considered: *Chlamydothrix, Clonothrix, Crenothrix, Detoniella, Didymohelix, Leptothrix, Leptotrichia, Nodofolium, Phragmidiothrix, Sphaerotilus.*

The following key give the characters which are believed by the writer to differentiate the genera which may be recognized.

Key to the genera of Chlamydobacteriaceae

1. Filaments not usually permanently attached.
   a. Filaments straight, or at least not twisted.....Genus 1. *Leptothrix*
   b. Filaments twisted.....Genus 2. *Didymohelix*
2. Filaments attached.
   a. Filaments unbranched.....Genus 3. *Crenothrix*
   b. Filaments show pseudodichotomous or false branching.
      (1) Swarm cells developed (motile conidia). Usually without a deposit of iron oxid in the sheath.....Genus 4. *Sphaerotilus*
      (2) Spherical, non-motile conidia. Usually with iron oxide. Genus 5. *Clonothrix*

Genus 1. **Leptothrix** Kuetzing, 1843, p. 198

Synonyms:

Chlamydothrix Migula, 1900, p. 1030
Leptotrichia Trevisan, 1879, p. 138
Detoniella Trevisan, 1889, p. 929

Filaments of cylindric colorless cells, with a sheath at first thin and colorless, later thicker, yellow or brown, becoming encrusted with iron oxide. The iron may be dissolved by dilute acid, whereupon the inner cells show up well. Multiplication is through the division and abstriction of cells and motile cylindric swarm cells. Swarm cells sometimes germinate in the sheath giving appearance of branching. Pseudodichotomous branching may occur.

The type species is *Leptothrix ochracea* (Leiblein) Kuetzing.

There has been considerable confusion relative to the appropriate designation of this genus. The name *Leptothrix* was created by Kuetzing for certain forms regarded as algae. The first species named was *L. ochracea*, the *Lyngbya ochracea* of Leiblein. Three other species were also described. *Leptothrix buccalis* an organism from the mouth, was named by Robin in 1852.

These facts have led to the development of three conceptions of the genus as follows:

1. *Leptothrix.* A genus of bacteria with the *L. ochracea* as the type.

2. *Leptothrix.* A genus of bacteria with *L. buccalis* as the type.

3. *Leptothrix.* A genus of algae. In this sense the genus has been recognized by many algologists. However, it may be noted that by recent writers (as in West's British Fresh Water Algae) the genus *Leptothrix* is made a synonym of *Lyngbya*. Many authors include *L. ochracea* with the algae.

Inasmuch as *Leptothrix ochracea* was definitely first named in this genus, it would seem to be entirely appropriate to make it they type of the genus.

It should be noted that this renders *Leptothrix* as applied to organisms such as *L. buccalis* quite invalid.

Genus 2. **Didymohelix** Griffith, 1853, p. 438

Synonyms:

Gaillonella Bory, 1823, in part
Gallionella Ehrenberg, 1838, p. 166, in part
Gloeotila Kuetzing, 1843, p. 245, in part
Spirophyllum ? Ellis, 1907, p. 516
Nodofolium ? Ellis, 1910, p. 321

Filament twisted, simple, or two filaments, twisted together. Young cells colorless, later yellow brown to rust red through deposition of iron. Simple filaments show no division into cells, even when iron is removed with acid and stain applied. Sheath not demonstrable.

The type species is *Didymohelix ferruginea* (Ehr.) Griffiths.

The generic name *Gallionella* was first used by Ehrenberg as a revised spelling of *Gaillonella*, a genus of diatoms created by Bory de St. Vincent. Ehrenberg included several true diatoms in the genus, together with this form, which he erroniously believed contained silicon, and to be a diatom. *Gallionella* is a valid diatom genus (or subgenus of *Meloseira* according to some authors), and should not be used as a generic name for bacteria.

Genus 3. **Crenothrix** Cohn, 1870, p. 130

Filaments unbranched, showing differentiation of base and tip, attached, usually thicker toward the tip. Sheaths plainly visible usually colorless, brownish from iron oxid in old filaments. Cells cyclindric to spherical. Multiplication by non-motile, spherical, conidia; cells dividing in 3 planes to form conidia.

The type (and only) species is *Crenothrix polyspora* Cohn.

Genus 4. **Sphaerotilus** Kuetzing, 1833, p. 385

Synonyms:

Cladothrix Cohn, 1875, p. 185

Attached colorless threads showing false branching, making a psuedodichotomy. Filaments consist of rod or oval cells, surrounded by a thin, firm sheath. Multiplication occurs both by non-motile and motile gonidia, the latter with a clump of flagella near one end.

Sphaerotilus natans Kuetzing is the type.

Genus 5. **Clonothrix** Schorler, 1904, p. 689

Filaments with false dichotomous or irregular branching, attached, with contrast of base and tip, tapering to the tip. Sheath always present, thin on young filaments, later becoming thicker and encrusted with iron or manganese. Multiplication by small non-motile gonidia of spherical form, formed from the disk shaped cells near tip by longitudinal division on rounding up.

The type species is *Clonothrix fusca* Schorler.

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EARLY INSTRUCTORS IN BACTERIOLOGY IN THE UNITED STATES

ADDENDA

E. G. HASTINGS AND C. B. MORREY

THE WORK OF WILLIAM TRELEASE

The recent paper[1] by Dr. D. H. Bergey on the "Early Instructors in Bacteriology in the United States" is of historic interest. It would seem that it should be made as complete as possible. The name of Dr. William Trelease is mentioned in connection with the development of bacteriology at the University of Wisconsin. No facts, however, are given as to the importance of his work in this connection.

Dr. Trelease came to the University of Wisconsin in 1881, leaving in 1885 to assume charge of the Missouri Botanical Gardens at St. Louis. In 1884 Dr. Trelease presented to the Academic Council of Harvard University as a doctor's thesis the results of some work he had done in the botanical laboratories at the University of Wisconsin. This work was published in the Studies from the Biological Laboratory of the Johns Hopkins University, Vol. III, No. 4, 1885 under the title "Observations on Several Zoogloeae and Related Forms."

Dr. L. H. Pammel, Wisconsin 1885, who is mentioned by Dr. Bergey as one of the pioneer instructors in bacteriology, received his first instruction in the subject from Dr. Trelease. Dr. Pammel states in a personal letter that Dr. Trelease introduced some work with the bacteria into his course in cryptogamic botany in the first semester of 1882–1883. Cultures were made on potato. The organisms of the mouth were studied, and the students were required to read Dr. Burrill's paper on "Bacteria," which is mentioned by Dr. Bergey. The work of Pasteur, Tyndall, Koch, Cohn, and others was discussed.

[1] J. Bact. 2, 595.

The first bacteriological apparatus was ordered by the University from Germany in 1885 at the request of Dr. Trelease. It did not arrive until after his departure, and it remained for Dr. E. A. Birge to develop formal courses in bacteriology at the University of Wisconsin. Dr. Trelease was, however, one of the first to give instruction in bacteriology in this country.

E. G. HASTINGS.

BACTERIOLOGY AT OHIO STATE UNIVERSITY

In the November, 1917, number of the Journal of Bacteriology there is an article by Dr. David H. Bergey on "Early Instructors in Bacteriology in the United States." I should like to add a few words concerning the teaching of bacteriology at the Ohio State University.

In the fall of 1885 Dr. H. J. Detmers came to the University as professor of veterinary surgery. He had previously been in the Bureau of Animal Industry associated with Dr. Salmon and also at the University of Illinois. He began doing bacteriological work as soon as he was established and the catalogue for 1886 lists a course in bacteriology for the spring term of one hour per week for senior veterinary students. By 1890 this had grown to a three hour course. Dr. Detmers did some research work on hog cholera during these years, and one of his students presented a paper at the Pittsburgh meeting of the American Microscopical Society, September 1, 1887, on the "Bacteriology of Foot Rot in Sheep," which paper was favorably commented upon in a number of journals throughout the country.

Not later than 1888 though I have not the exact date at hand, courses in bacteriology were offered to medical students in Starling Medical College, now a part of the University.

C. B. MORREY.